SURVEY FOR *CERATOCYSTIS FIMBRIATA* ON *SYNGONIUM* SPECIES, HOST RANGE TEST AND MOLECULAR CHARACTERIZATION OF THE ISOLATES COLLECTED

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ABSTRACT

Rapid Ohia Death (ROD) caused by *Ceratocystis lukuohia* and *C. huliohia* is killing several thousand hectares of ohia forests in the Island of Hawaii. *Ceratocystis lukuohia* is closely related to *C. fimbriata* from *Syngonium* in Hawaii based on phylogenetic analysis. However, the distribution and diversity of the *C. fimbriata* from *Syngonium* is not known. Surveys were conducted on the Islands of Oahu, Hawaii, Maui, and Kauai to determine the distribution of *C. fimbriata* in *Syngonium*. *Ceratocystis fimbriata* was recovered from the samples collected from two commercial nurseries in Hilo, Hawaii. *Ceratocystis fimbriata* was not isolated from the samples collected from Oahu and no *Syngonium* plants having typical symptoms of *C. fimbriata* were obtained from Maui and Kauai. Koch's postulate was established on *Syngonium* with the *C. fimbriata* isolates collected from *Syngonium*. However, Ohia plants inoculated with *C. fimbriata* from *Syngonium* showed no symptoms after three months post inoculation even when the optimal conditions necessary for infections were provided. *Ceratocystis fimbriata* isolates 3401, 3459 and 3466 were not different to each other in terms of radial growth at 15°C, 20°C, 25°C and 30°C as revealed by analysis of variance at 0.05 level of significance but statistical analysis of the dimensions of perithecia, asexual and sexual spores at the same level of significance showed that *C. fimbriata* isolates 3401, 3421, 3459 and 3466 were significantly different . Fifteen *C. fimbriata* isolates collected from Hilo, Hawaii during the survey were clonal. They showed no sequence diversity for Internal Transcribed Spacer (ITS) rDNA region, Beta-tubulin 1(*Bt1*), Transcription elongation factor-1 alpha (*tef1*), Guanine Nucleotide binding protein subunit beta-like protein (*ms204*) and second largest subunits of RNA polymerase II (*rpb2*). *Ceratocystis fimbriata* collected from *Syngonium* in this study was identical for all five genes to *C. fimbriata* collected from *Syngonium* in the 1980s and these isolates grouped together in the phylogenetic tree with strong branch support.

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CHAPTER 1

IMPORTANCE OF OHIA AND ITS MAJOR DISEASES

Introduction to Ohia and its Importance to the Hawaiian Islands

The Ohia tree (*Metrosideros polymorpha*) is the dominant component of the Hawaiian rainforest and makes up ~80% of the trees in the existing forest in the Island of Hawaii (Mueller-Dombois and Loope 1990). Among 400, 000 hfa of ohia and ohia-koa forests in Hawaii, the Island of Hawaii alone has more than 287, 700 ha of these kind of forests (Petteys et al. 1975). Ohia grows from sea level to 2500 m altitude in Hawaii (Aradhya et al. 1991). Ohia forest is the foundation to the Hawaiian rainforest ecosystem which consists of many flora and fauna among which 90% are only found in the Hawaiian Islands (Loope 2016). Ohia forests are home to 10, 000 native invertebrates among which 90% are endemic (Mueller-Dombois et al. 2013). Invertebrate populations closely associated with ohia are composed of insects, mites, spiders, snails, slugs, and butterflies. For example, within 11 sites in the *M. polymorpha* forest 495 endemic species of arthropods were found on Hawaii Island (Gruner 2004). Ohia provides food and habitat to 22 species of forest birds including the endemic and endangered honey creepers (Loope 2016). Some of the endemic birds found in the Hawaiian Islands are *Himatione sanguinea*, *Vestiaria coccinea*, *Chlorodrepanis virens*, *Hemignathus wilsoni*, *Chasiempis sandwichenensis* and *Myadestes obscurus* (Mueller-Dombois et al. 2013). *Chasiempis sandwichenensis* and *Myadestes obscurus* are listed as threatened species by the World conservation union (IUCN).

Ohia forests hold significant cultural importance in Hawaii. Ohia is considered the physical manifestation of Kū, one of the four major Hawaiian deities, as well as other deities such as, that of hula (Laka), and the volcano (Pele). Flowers of ohia symbolically represent the fire of the goddess Pele (Gon 2012). Ohia wood is used to create the most sacred components of the temples like god figures (ki‘i akua) and the offering platform (Lele) (Gon 2012). Ohia wood is also part of the ‘ānul‘u (oracle tower)

and ohia boles are used in temple enclosures (pā) (Mueller-Dombois et al. 2013). In addition, the flowers of ohia are used for making lei (Gon 2012). Young flowers, leaf buds and aerial roots of ohia trees are used in traditional medicine as it is believed to act as a tonic, appetizer, and disinfectant (Mueller-Dombois et al. 2013). The strong wood of ohia trees is preferred for war weapons like lāau (war clubs) and pāhoa (daggers) and agricultural tools like ʻōʻō and koʻI (adzes) (Mueller-Dombois et al. 2013). Wood is used for making cooking containers and utensils and incorporated into different structures of homes (Gon 2012). Ohia trees help in water conservation since overall water use for ohia is less than invasive species (Cavaleri et al. 2014). In Hawaii, the trade winds capture the moisture evaporating from the ocean and bring it to the islands. Part of the moisture is captured by the leaves of the ohia plants via condensation forming the water droplets, which runs down along the leaves, branches and stem of the plants. Ohia trees also gather the mist that form rain. This is crucial to replenish the water in the forest.

Major Diseases of Ohia (*Metrosideros polymorpha*)

Ohia Rust

A cultivated ohia plant submitted for diagnosis at Agricultural Diagnostic Service Center (ADSC) at the University of Hawaii at Manoa on April 2005 was found to be infected with rust (Uchida et al. 2006). Based on the urediniospore morphology and Internal Transcribed Spacer (ITS) region the causal organism was identified as *Puccinia psidii*. Even though *Puccinia pisidii* has two-celled teliospores (major characteristics of Pucciniales), it formed a well-supported clade separated from other species of genus *Puccinia* and family Pucciniaceae (Beenken 2017). *Puccinia psidii* was recently renamed as *Austropuccinia psidii* and moved to a new family, Sphaerophragmiaceae (Beenken 2017). A few months after the rust was observed on ohia, it was also observed on other Myrtaceae hosts in Hawaii including nonnative *Syzygium jambos* (L.), endangered *Eugenia koolauensis* and *E. reinwardtiana* (Uchida et al. 2006) and was seen in Allspice in 2008 (Kadooka 2010). *Austropuccinia psidii* was originally reported in Brazil on guava (*Pisidium pomiferum*) in 1884 and on eucalyptus in 1994 and is now reported from South

America, Central America, the Caribbean, Florida (Coutinho et al. 1998) and Japan (Kawanishi et al. 2009). It has a very broad host range and has been reported to infect 56 genera and 224 species of plants (Beenken 2017). All of the isolates of *Austropuccinia psidii* from different Myrtaceae in Hawaii had the same genotype and were closely related to isolates collected from Florida; one Florida isolate had 95% similarity to the Hawaiian isolates suggesting that *Austropuccinia psidii* might have been introduced to Hawaii from Florida recently (Zhong et al. 2011). Kadooka (2010) reported the similarity of the Hawaii isolates to isolates of *A. psidii* from Myrtle in California. Currently, *A. psidii* is known to infect 33 plant species from the Myrtaceae family (Silva et al. 2014) among which five are endemic to Hawaii (Chris Kadooka, personal communication).

At present, the pathogen has been observed state-wide causing infections on plants growing at altitudes of 1200-1500m and in areas with mean annual precipitation ranging between 750 mm- 5000 mm in Hawaii (Loope and Uchida 2012). Fortunately, at present, the damage caused by *Austropuccinia psidii* to ohia trees is minimal; however, rose apple (*Syzygium jambos*) has suffered severe losses and crown dieback has been observed at the landscape scale dying throughout the state (Loope and Uchida 2012). In Hawaii, winter months are very conducive to infection because of the mild temperatures and increased wetness favoring disease, and the potential for outbreaks of a strain capable of severely damaging ohia like the current strain damaging rose apple cannot be overlooked (Loope and Uchida 2012). Twenty-three multi locus genotypes of *Austropuccinia psidii* were found among the isolates collected from Brazil, Costa Rica, Jamaica, Mexico, Puerto Rico, Uruguay and USA (Stewart et al. 2017). Three *Austropuccinia psidii* isolates from *Eucalyptus grandis, E. urophylla* X *E. grandis* and, *Myrciaria caulifora* in Brazil were highly virulent on six populations of ohia from Hawaii (Silva et al. 2014). There is a great threat of introduction of these virulent strains to Hawaii.

Rapid Ohia Death

Between 2009 - 2010 landowners in the Puna District of Hawaii Island began to notice yellowing and browning of ohia trees followed by sudden death of those trees within a few days to weeks. Rapid dying of ohia trees became more pronounced as 2,400 ha of ohia forest began dying in patches, progressing from Kalapana to Hilo in 2014. The disease was eventually detected in Kona by the end of 2015 (Loope 2016). Although the beginning of this disease is unknown, based on recent aerial surveys in 2018, about 55,000 ha of ohia forest currently show symptoms of Rapid Ohia Death (ROD) on the Island of Hawaii (Friday, J. B. 2018). Among the affected forest area, 1,600 ha in 2012 and 6,403 ha in 2014 had canopy mortality of >10%, whereas 3,824 ha of the remaining ohia forests had less than 10% canopy mortality at that same time (Mortenson et al. 2016). This data was obtained using the remote sensing imagery used to monitor the disease on the Big Island. Aerial photographs taken with the help of helicopters were divided into 100m x 100m grid cells which were visually inspected to categorize the mortality based on the leafless fine branches left on the canopy after the leaves had turned brown and fallen (Mortenson et al. 2016). Initially, the etiology of the disease was identified as *Ceratocystis fimbriata* (Keith et al. 2015). *Ceratocystis fimbriata* is a species complex and potentially contains many undescribed species (Baker et al. 2003). A recent study identified two new species of *Ceratocystis* within the *C. fimbriata* species complex responsible for ROD (Barnes et al. 2018). They were named *C. lukuohia* (formerly referred to as species A) and *C. huliohia* (formerly referred to as species B). *Ceratocystis lukuohia* is the more aggressive of the two species and kills trees in a shorter period of time, whereas *C. huliohia* is a canker pathogen and symptoms require more time to develop (Barnes et al. 2018). Typically, entire crowns of infected trees turn yellow, then quickly turn brown within weeks and the brown leaves remain attached to the stems (Barnes et al. 2018). The fungus causes dark brown to black staining of the xylem (Barnes et al. 2018) due to the color of the fungal mycelium and spores (Lisa Keith, personal communication). Although there are areas on Hawaii Island with almost complete ohia mortality, there is no clear pattern of spread. Healthy ohia trees surrounding infected trees are commonly observed (Loope 2016).

The current mortality of ohia forests is different from the earlier ohia decline that occurred in the 1960s and 1970s because ROD is observed in areas where the earlier decline was not reported and trees of all ages are affected by ROD (Mortenson et al. 2016). Ohia decline in the 1960s and 1970s occurred in cohorts and was associated with the age of the volcanic substrates they were growing on (Yoshiko and Mueller-Dombois 1995). Weather disturbances along with abiotic stresses like nutrient deficiency, poor soil drainage on the old volcanic substrate might have caused the death of the ohia trees in cohorts (Yoshiko and Mueller-Dombois 1995).

Biology and Morphology of *C. fimbriata*

Variation in terms of colony type is observed among the isolates of *Ceratocystis*. Webster and Butler (1967) studied the morphology of 45 isolates of *Ceratocystis* from 11 different hosts from diverse geographic areas and grouped them into three groups based on the colony color and distribution of perithecia. First group consisted of the isolates from coffee (*Coffeea arabica*), plane tree (*Platanus sp.*) and, oak (*Quercus ellipsoidalis*) that formed hyaline to light olive brown colonies on malt extract agar with perithecia distributed uniformly on the culture (Webster and Butler 1967). The isolates from aspen (*Populus tremuloides*), apricot (*Prunus armeniaca*), prune (*Prunus domestica*), peach (*Prunus persica*) and almond (*Prunus amygdalus*) were in the second group and had brown to dark olive colored colonies and perithecia were produced in clumps or in concentric rings (Webster and Butler 1967). Isolates from cacao and sweet potato formed third group and produced perithecia scattered into small clumps or singly present and colonies were smoky grey to dusky olive green (Webster and Butler 1967). *Ceratocystis fimbriata* grows optimally between the temperature of 20-25°C (Wingfield et al. 1996). Webster and Butler (1967) reported the optimal temperatures to be 24-27°C. Thiamine is necessary for *Ceratocystis* isolates to produce perithecia in culture (Webster and Butler 1967). Vegetative growth is sparse in culture deficient in thiamine (Webster and Butler 1967).

Sexual spore

Ascospores are the sexual spores of ascomycete fungi and are produced in the fruiting body called an ascocarp or perithecia. The perithecia of *C. fimbriata* consists of three parts: a swollen base, elongated neck and ostiolar hyphae. Ascocarps are made up of pseudoparenchyamatic tissue composed of irregularly arranged evanescent asci at the base (Upadhyay H.P. 1981). Ascospores are embedded in a mucilaginous substance and form a cream colored droplets at the tip of the ascocarp (Valdetaro et al. 2015). In nature, ascocarps are formed on the surface of discolored, infected wood (Barnes et al. 2003) or on the black rot of infected colocasia corms (Harrington et al. 2005). In culture, the perithecial bases are superficial or partially embedded in the media (Oliveira et al. 2015). Bases of the perithecia are dark in color (Wingfield et al. 1996; Xu et al. 2011) and round to oval (Upadhyay H.P. 1981). Ostiolar hyphae are hyaline and non-septate (Valdetaro et al. 2015) in the in the tip of perithecial neck. *Ceratocystis fimbriata* produces typical hat shaped ascospores containing a brim of the gelatinous substance going continuously around the ascospore (Fateh et al. 2006). Ascospores are formed in pairs and are connected brim to brim in the developing asci (Webster and Butler 1967). Asci are mostly spherical to sub-spherical, but can also be clavate, and contain 8 ascospores (Upadhayay H.P. 1981). The dimensions of the perithecia base is 120-260 µm in diameter while perithecia neck range 10.8 µm at the top to 33.7 µm at the base, whereas the ascospores are typically 3-8 µm in diameter (Webster and Butler 1967). Some variation in ascocarp and ascospore morphology has been observed among the isolates from different groups within the *C. fimbriata* complex. The North American clade consisting of aspen, prunus, hickory, and oak isolates have smaller ascospores (3.5-6.5 µm long and 3-5 µm wide) than the normal size range (5.5–7.0 µm long x 3.5–5.5 µm wide) for *C. fimbriata*; most isolates of the North American clade have a collar at the point where the perithecial neck emerges from the base, which is absent in the Latin American clade (Johnson et al. 2005). The ascospore dimensions for *C. lukuohia* and *C. huliohia* are (4.5–)5–6 × 2.5–3(–3.5) µm and (3.5–)4.5–5(–5.5) × (1.5–)2–2.5(–3) µm, respectively (Barnes et al. 2018). Temperature and media type profoundly affect the formation of ascospores (Upadhyay H.P. 1981). No perithecia were produced by *C. lukuohia* and *C. huliohia* at 10°C and 30°C (Barnes et al. 2018).

Perithecia
Valdetaro et. al, 2015

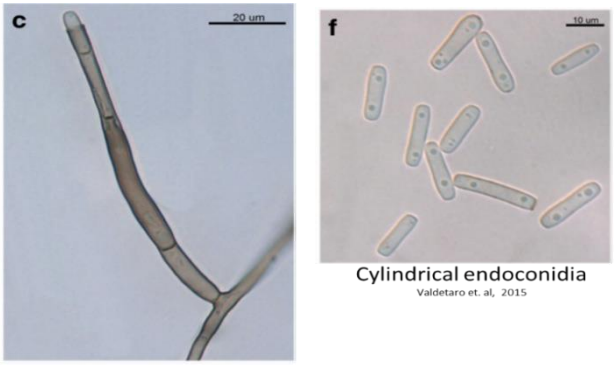
Ascospores
Valdetaro et. al, 2015

Asexual Spores

Ceratocystis fimbriata produces three kinds of asexual spores: cylindrical endoconidia, doliform endoconidia and thick-walled spores called aleurioconidia or chlamydospores. Conidia are produced on conidiophores bearing conidiogenous cells, determinate or branched percurrently (new apex originate from previous apex) or sympodically (development of conidia on zig-zac or geniculate rachis) and conidia are blastic (recognizable development of conidia before the septation occurs in conidiophore) which can be enteroblastic (when only the inner walls of the conidium bearing cells are involved in conidiogenesis) and holoblastic (when the wall of the conidium is continuous with the cell that produced it) (Upadhyay H.P. 1981).

Cylindrical Endoconidia

Ceratocystis fimbriata isolates from most hosts, including sweet potato (*Ipomoea batatus*), cacao (*Theobroma cacao*), coffee (*Coffeea arabica*), plane tree (*Platanus sp*), aspen (*Populus tremuloides*), oak (*Quercus ellipsoidalis*), apricot (*Prunus armeniaca*), prune (*Prunus domestica*), peach (*Prunus persica*) and almond (*Prunus amygdalus*) produce cylindrical endoconidia in phialides (Webster and Butler 1967). Conidiophores are hyaline to sub hyaline, septate and borne singly or in clusters from both aerial and subsurface mycelium with conidia produced in chains. Cylindrical, hyaline, single celled conidia range in size from 6.8 to 36.9 µm long and 3.2-6.9 µm in diameter (Webster and Butler 1967). On malt extract agar (MEA), average dimensions for cylindrical endoconidia for *C. lukuohia* and *C. huliohia* are 16.0 x 4.0 µm and 20.0 x 3.5 µm, respectively (Barnes et al. 2018).



Cylindrical endoconidiophore
Valdetaro et. al, 2015

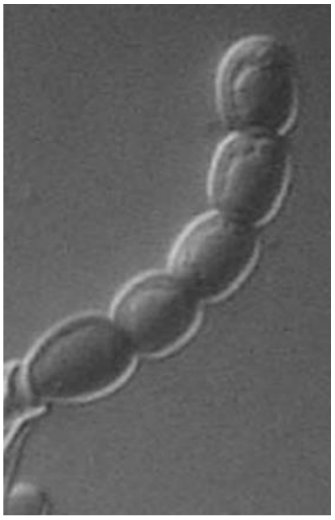
Cylindrical endoconidia
Valdetaro et. al, 2015

Doliform Endoconidia

In contrast to the narrow cylindrical endoconidia, doliform endoconidia are produced in the wide mouth phialides which are shorter then the phialides producing cylindrical endoconidia (Johnson et al. 2005; Webster and Butler 1967). Doliform conidia are initially hyaline then gradually change to subhyaline to brown as they mature (Webster and Butler 1967) and are observed aggregating around the perithecial bases (Johnson et al. 2005). Webster and Butler (1967) observed the average dimensions to be 5.5- 10.8 µm long and 4.9- 9.6 µm wide for isolates form from sweet potato, cacao, coffee, plane tree, aspen, oak, apricot, prune, peach, and almond.

Not all isolates of *C. fimbriata* produce doliform conidia; however, they are produced by isolates from aspen, oak, stone fruits (Webster and Butler 1967); aspen, almond, hickory, oak (Johnson et. al 2005); sycamore (Engelbrecht and Harrington 2005); pomegranate (Xu et al. 2011) and *Eucalyptus grandis* (Barnes et al. 2003). *Ceratocystis fimbriata* infecting *Syngonium* and *C. lukuohia* and *C. huliohia* infecting ohia also produce doliform conidia.

Wide mouth phialide
Johnson et al. 2005



Doliform endoconidia
Johnson et. al, 2005

Aleurioconidia or Chlamydospores

Aleurioconidia, also referred to as chlamydospores, are thick-walled pigmented conidia with smooth or rough surfaces, oval to subglobose in shape; produced singly or in chains and borne on simple or branched conidiophores or embeded in the host surface in nature (Webster and Butler 1967). Aleurioconidia are 8.7-19.6 µm long and 6.2- 16.1 µm wide (Webster and Butler 1967) for isolates from sweet potato, cacao, coffee, plane tree, aspen, oak, apricot, prune, peach and almond. Aleurioconidia are also produced by *C. lukuohia* and *C. huliohia* from ohia in Hawaii (Barnes ae al. 2018). On MEA, average dimensions of aleurioconidia produced by *C. lukuohia* and *C. huliohia* are 14.0 x 10.0 µm and 13.0 x 10.0 µm, respectively (Barnes et al. 2018).

Aleurioconidia

Valdetaro et. al, 2015

Infection and Colonization

Ceratocystis fimbriata is known as a wound pathogen and infects wounds (Barnes et al. 2003; Kile 1993; Roux et al. 2004; Van Wyk et al. 2005). Fresh wounds 2-5 days old are necessary for succesful infection of oak and *Platanus* by *C. fagacaerum* and *C. platani*, respectively (Kile 1993). Infection may occour through prunning wounds, wounds formed on roots growing along compact soils or wounds made by stem boring insects (Panconesi 1981). On eucalyptus, wounds formed by the recent prunning are the most ideal sites for infection and streaking symptoms start and progress inward from the prunning sites, whereas unpruned *Eucalyptus globulus* remain disease free (Barnes et al. 2003). Unwounded plants can defend themselves, but not when a tinniest of wounds are present (Panconesi 1981). In the Brazilian State of Minas Gerais, *Ceratocystis* populations have high genetic diversity suggesting it is native to this area

(Ferreira et al. 2011); soil borne inoculum causes infections (natural infection, wounds not necessarily required) in eucalyptus plantations in Minas Gerais (Ferreira et al. 2011).

Ceratocystis platini germinates in the lesion, grows and moves along the medullary ray cells reaching the pith to finally colonize the xylem elements; however, it is unable to modify the walls of the xylem vessels (D'Ambra et al. 1977). Sometimes *C. platani* can move within the tissue and emerge from unhealed lesions (D'Ambra et al. 1977). *C*eratocystis *platani* spores make their way to fresh wounds on infected pruning tools, on the surface of animals (including insects, rodents, and birds), or are carried by rain or hail (Panconesi 1981).

The ability of the fungus to colonize the host tissue varies according to the resistance of the host. *Ceratocystis fimbriata* extensively colonizes the parenchyma and xylem vessels and produces many thick walled chlamydospores in the vessels, growing in radial directions from the pith to again reach the vessels; however, in moderately resistant and resistant cultivars, xylem vessels remain uncolonized and free of occulsion by the deposition of gums, tyloses and phenolic compounds (Araujo et al. 2014). Few chlamydospores were observed and *C. fimbriata* rarely reached the pith in moderately resistant and resistant cultivars (Araujo et al. 2014). It is argued that all stem tissue of mango can be colonized by the fungus, so it cannot be the exclusive vascular pathogen as mentioned in some literature (Araujo et al. 2014).

Symptoms

On woody hosts, *C. fimbriata* causes wilt and cankers whereas, it causes black rot of corms (*Colocasia*), aerial roots (*Syngonium*), tubers (Sweet potato) and stems (*Syngonium*) on herbaceous hosts. Dark streaking and discolouration of the vascular bundle are characteristic symptoms of *C. fimbriata* (Barnes et al. 2003; Fateh et al. 2006; Masood et al. 2011; Roux et al. 2000) on woody hosts. Rapid wilting of the foliage causes the leaves to remain attached to the stem (Barnes et al. 2003) which is followed by necrosis and dieback (Barnes et al. 2003; Roux et al. 2000). Gummosis and bark splitting

occurs in eucalyptus and mango, and small canker can also be observed in the bark of these hosts (Barnes et al. 2003; Masood et al. 2011; Roux et al. 2000). In roots and corms, *C. fimbriata* causes black rot (Harrington et al. 2005; Huang et al. 2008). Black to brown discolored areas extending a few milimeters into the skin surface with occasional pink to orange internal discoloration were observed in the corms of *Colocassia esculenta* (Harrington et al. 2005).

Two new species from the *Ceratocystis fimbriata* species complex (*C. lukuohia* and *C. huliohia*) were recently reported to cause rapid death of ohia in Hawaii (Barnes et al. 2018; Keith et al. 2015). Trees die rapidly within weeks to a few months once wilt symptoms are observed, and leaves remain attached to the branches. Trees of all age group are affected by the disease (Mortenson et al. 2016). Dark brown to black xylem discoloration can be observed in infected trees (Keith et al. 2015) which is caused by the accumulation of fungal mycelium and aleurioconidia is the plant tissue.

Dispersal

Ceratocystis fimbriata is associated with different bark boring coleptera like Scolytidae and Cerambycidae or sap feeding beetles like Nitidulidae (Craighead and Nelson 1960). Beetles are attracted to the declining trees and ascospores embedded in mucilage protruding from perithecia get attached to the hydrophobic exoskeleton of the insects (Loope 2016). *Ceratocystis* spp. produce a fruity odor that attracts insects to facilitate dispersal (Kile 1993; Barnes et al. 2003). Kamgan Nkuekam et al. (2012) isolated *C. fimbriata* from nitidulid beetles in the genus *Brachypeplus* from eucalyptus in Australia. Masood and Saeed (2012) not only isolated *C. fimbriata* from the beetles, but also proved that the beetles, *Hypocryphalus mangiferae* were transmitting the pathogen to a healthy mango tree which had a cloth mesh attached to its trunk where the infected beetles were released. The same species of beetle was identified to be an effective vector of mango decline disease in Brazil by Ribiero (1980) and in Oman it was found to be associated with the mango decline pathogen *C. manginecans* (Al Adawi et al. 2013). Bark boring beetles not only directly transmit the disease from an infected tree to a healthy tree, but also

bore galleries and produce frass which contains fungal spores. The spores in the frass are dispersed long distances through the air. Masood and Saeed (2012) were able to isolate *C. fimbriata* from the frass produced by infected bark beetles inoculated into a healthy tree. Though insects vector *C. fimbriata* moves within short distances, but humans are responsible for spreading the pathogen across long distances. The pathogen can be moved in infected corms, tubers, cuttings, wood, and crating material made from the wood of infected trees (Baker et al. 2003; Harrington et al. 2015; Thorpe et al. 2005). Though the role of beetles to directly transmit inoculum from infected ohia trees to healthy trees is unknown, some of the wood boring ambrosia beetles including *Xyleborinus saxesenii, Xyleborus ferrugineus, Xyleborus affinis* and *Xyleborus simillimus* have been identified to be associated with ohia and 10% of the ambrosia beetles collected from infected stand have been found to carry *C. lukuohia* (Loope 2016). In addition to insects and windblown frass carrying viable spores, *C. fimbriata* could be moving in contaminated soil and water, contamination pruning tools (Loope 2016). Feral cats and pigs, birds, humans (wood trade, shoes, and other plant products such as leis) and root grafting could be other possible ways by which the pathogen is moving across Hawaii Island (Loope 2016).

Host Range and Distribution of *C. fimbriata*

Ceratocystis fimbriata has a broad host range that includes annual plants and perennial trees. Except for some plants in the family Araceae, all additional hosts of *C. fimbriata* are dicots. The *Ceratocystis fimbriata* species complex includes many cryptic species specialized to different hosts. The dicot hosts of the pathogen include *Ipomea bataus, Eucalyptus* spp, *Citrus* spp, *Carica* spp, *Acacia* spp, *Ficus* spp, *Heava spp, Pimenta dioica, Prunus amygddalus, Punica granatum, Quercus ellipsoidalis, Theobroma cacao* and many more. The monocot hosts of the pathogen include *Syngonium podophyllum, Colocasia* spp *and Xanthosoma* spp. *Ceratocystis fimbriata is* a known pathogen of *Colocasia esculanta, Xanthomoma xaggittifolium, Xanthosoma batavine* and some unknown species of *Xanthosoma* (Thorpe et al. 2005). In Hawaii, besides ohia, *C. fimbriata* is present on sweet potato where it causes black rot of

tubers, on taro where it causes black rot of taro corms, as well as on *Syngonium* and causes stem, leaf and root rot (Thorpe et al. 2005; Uchida and Aragaki 1979). The *Syngonium* strain found in Hawaii is identical to those found in Florida, California, Australia, and Brazil in ITS sequence and is probably native to the Caribbean (Thorpe et al. 2005). *Ceratocystis fimbriata* infecting *Syngonium* is of special interest because molecular analysis of the *Syngonium* strains and the newly discovered ohia strains revealed that these are closely related pathogens within the C. *fimbriata* species complex (Barnes et al. 2018). *Ceratocystis platani*, causing canker stain disease of plane trees is also closely related to *C. fimbriata* from *Syngonium*. *Ceratocystis platani* from plane trees from North Carolina and *C. fimbriata* from *Syngonium* from Hawaii grouped together in phylogenetic analysis of ITS and the MAT1-2-1 gene (Li et al. 2017).

Host Specialization

Ceratocystis fimbriata populations can be broadly divided into three clades from Asia, North America and Latin America (Harrington 2000). Within these clades are populations with unique genotypes which are specialized to infect unique hosts. Cacao, sweet potato and sycamore isolates are uniquely pathogenic to the host they were isolated from and cacao and sycamore isolates form a well supported lineage in the parsimony analysis based on ITS sequence (Baker et al. 2003). Geographically isolated populations can develop the ability to uniquely infect a native host plant and these populations with unique genotypes and host specialization may represent undescribed species (Baker et al. 2003). Isolates from cacao and sycamore were later recognized as *C. cacaofunesta* and *C. platini* by Engelbrecht and Harrington (2005) due to their host specialization, morphology, intersterility and unique ITS genotypes. Strong host specialization was observed in Aspen (*Populus tremuloides*) and Hickory (*Carya* spp.). Johnson et al. (2005) recognised Aspen and Hickory isolates as *C. populicola* and *C. caryae* based on interfertility, host specialization, unique genotype in the ITS and alloenzyme analysis. Pathogenicity tests on the native and exotic hosts of different families in Brazil failed to show the conspicuous pattern of host specialization of

the Brazilian isolates from *Gemelina arborea*, *Ficus carica*, *Colocasia esculenta* (inhame), *Eucalyptu*s spp. and mango (*Mangifera indica*). There was no correlation between where the plants came from and agressiveness of the pathogen (Harrington et al. 2011). Isolates from aroids appear to have specialized to infect plants in the family araceae and not other woody plants (Thorpe et al. 2005). There appears to be some sort of host specialization within the isolates of *C. fimbriata* collected from aroids (Thorpe et al. 2005). *Ceratocystis fimbriata* isolates from *Syngonium* and *Xanthosoma* are more pathogenic to *Syngonium* compared to *Colocasia* isolates and *Colocasia* isolates cause more petiole discoloration in *Colocosia* compared to *Syngonium* isolates (Thorpe et al. 2005). *Syngonium* and *Colocasia* isolates were identical except for 2 base pairs in their ITS sequences (Thorpe et al. 2005).

Genetic Diversity of *Ceratocystis fimbriata* Isolates from *Syngonium*

ITS sequences from *Syngonium* isolates of *Ceratocystis* from Hawaii, Florida, Brazil, and Australia are identical and are most closely related to *Xanthosoma* isolates from Costa Rica, Cuba, Puerto Rico and Dominican Republic (Thorpe et al. 2005). Their ITS sequences only differed by 2 base pairs. These findings from ITS phylogeny were supported by multigene phylogeny where *C. fimbriata* isolates from *Syngonium* were most closely related to *Xanthosoma* isolates from Costa Rica and Cuba and very close to *C. platani* isolates from plane and *C. lukuohia* from ohia (Barnes et al. 2018). *Syngonium* isolates from Hawaii and Florida were also identical in the phylogenetic analysis involving five genes (Barnes et al. 2018).

Management

Genetic Resistance

Growing resistant cultivars is the most suitable method to manage the disease of forest species which are grown for commercial use (Guimarães et al. 2010). Cultivating resistant plants has been the major strategy to control *Ceratocystis* wilt of *Mangifera indica* (Ribiero et al. 1995), *Coffeea* (Castilla 1982),

Crotolaria (Ribiero et al. 1997) and eucalyptus (Rosado et al. 2016). Guimarães et al. (2010) evaluated the resistance of *Eucalyptus pelita* to *C. fimbriata*; 16 clones among 23 used were resistant to *Ceratocystis fimbriata* and significantly indifferent to the resistant control used. Quantitative Trait Loci (QTL) controlling resistance to *C. fimbriata* has been identified to facilitate marker based selection of resistant cultivars in eucalyptus (Rosado et al. 2016) and cacao (Santos et al. 2012). The mechanism of inheritance of resistant genes has also been studied in mango (Arriel et al. 2016) and eucalyptus (Rosado et al. 2010). Resistance to *C. fimbriata* in mango is polygeneic (Arriel et al. 2016) and five quantitative trait loci (QTLs) governing resistance to *C. fimbriata* were identified in eucalyptus (Rosado et al. 2010).

Chemical Control

Ceratocystis fimbriata caused major loss in sweet potato production in the 1990's, but historically it had been well controlled using integrated pest management. The disease recently reemerged in the United States (Scruggs et al. 2017). Efficacy of 8 different fungicides, including difenoconazole, fludioxonil, thiabendazole, dicloran, azoxystrobin, pyraclostrobin, fenamidone, and fluazinam were evaluated in vitro (Scruggs et al. 2017). Difenoconazole and thiabendazole not only reduced mycelial growth in vitro, but also on tubers receiving post-harvest treatment with the same fungicides (Scruggs et al. 2017).

Cultural Control

Crop rotation, planting of disease free planting materials, curing for wound healing and skin hardening were some of the cultural components of an integrated approach to manage black rot of sweet potato in the 1900's (Scruggs et al. 2017). Adoption of indispensable pruning during the favorable months of the year was suggested as one of the strategies to prevent *C. platani* infections (Panconesi 1981). Felling of severely infested trees is an additional strategy to prevent the spread of the pathogen in surrounding areas (Panconesi 1981).

Sanitation

Destruction of sawdust from felled trees, disinfection of stumps, surrounding soil, pruning equipment and pruning wounds helps to manage canker stain of plane trees; Benomyl (80g/hl) or ethyl alcohol can be used for disinfection (Panconesi 1981). Disinfection of pruning tools with 10% chlorine bleach or 70% isopropanol is recommended to prevent the spread of Rapid Ohia Death (Loope 2016).

Impact of Rapid Ohia Death

Loss of ohia due to the two new species of *Ceratocystis* will cause loss of food and habitat for many flora and fauna inhabiting ohia forests. There is a greater risk associated with complete loss of endangered plants and animals. Many closely related bird species in the Hawaiian honeycreeper family are adapted to feed on ohia (Buermeyer et al. 2008). Two birds species, Po'ouli (*Melamprosops phaeosoma*) and 'O'o'a 'a (*Moho braccatus*) became extinct within the last twenty years (Mueller-Dombois et al. 2013). Habit destruction, avian diseases (avian malaria and avian pox) and predation by cats and mongooses are some of the causes for the extinction of these species. Loss of ohia threatens the survival of remaining species because loss of ohia will also cause the loss of their habitat and food resources. Loss of dominant trees of the Hawaiian forest can have serious long-term consequences (Petteys et al. 1995) like drying of the watershed and extinction of biodiversity. Ohia forest are fragile and can be displaced by alien tree invaders like strawberry guava (*Psidium cattleyanum)*, faya tree (Morella *faya*), albizia (*Falcataria moluccana*), ironwood (*Casuarina equisetifolia*) and velvet tree (*Miconia calvescense*) (Mueller-Dombois et al. 2013). Alien birds such as *Streptopelia chinensis, Acridotheres fristis, Zosterops japonica* and *Carpodaceus mexicanus* feed on fruits of strawberry guava and aid in dispersal of seeds throughout the forest (Mueller-Dombois et al. 2013). Stand level dieback or death of large number of ohia plants of the same age facilitates the establishment of invasive species (Gregory et al. 1998). Besides threatening the survival of flora and fauna, impacts of ROD on the watershed and fresh water resources can be devastating.

Objectives

1. Conduct surveys on Oahu, Hawaii, Maui and Kauai to determine if *C. fimbriata* is present on *Syngonium*.

2. Test the pathogenicity of *C. fimbriata* isolates collected from *Syngonium* on *Syngonium* and ohia.

3. Study morphological (conidia, ascospores, aleurioconidia and perithecia) and cultural characteristics of *C. fimbriata* isolates collected from *Syngonium*.

4. Conduct molecular characterization of *C. fimbriata* isolates from *Syngonium.*

CHAPTER 2

SURVEY FOR *CERATOCYSTIS FIMBRIATA* ON *SYNGONIUM*

Introduction

Ceratocystis fimbriata was reported from *Syngonium podophyllum* in the 1970s from Hawaii causing leaf spots and rotting of the aerial roots, nodes and stems of *Syngonium* plants (Uchida and Aragaki 1979). On water agar plates, *Syngonium* isolates of *C. fimbriata* start as hyaline mycelium and eventually turn dark with age on V8 agar. *Ceratocystis fimbriata* produces sexual (ascospores) and asexual spores (cylindrical conidia, doliform conidia and aleurioconidia) in culture. *Ceratocystis fimbriata* infecting *Syngonium* is of special interest because molecular analysis revealed that *C. fimbriata* from *Syngonium* is genetically close to *C. lukuohia* causing Rapid Ohia Death on the Island of Hawaii (Barnes et. al 2018). *Ceratocystis fimbriata* from *Syngonium* is an understudied pathogen and the possibility that the strains that affect this host may be equally pathogenic on ohia, is of serious concern. The extent of its presence and genetic diversity in Hawaii is unknown. Thus, it is crucial to characterize *C. fimbriata* from *Syngonium*.

Materials and Methods

Survey for *Ceratocystis fimbriata* on *Syngonium*

The island of Oahu and three nurseries on the island of Hawaii were surveyed for the presence of *C. fimbriata* on *Syngonium*. Twenty-two different locations on Oahu, including commercial nurseries, botanical gardens, one arboretum and retail garden centers were surveyed. Leaves, petioles, stems and nodes of the plants of different species and cultivars of *Syngonium* found in the survey locations were

carefully examined for the presence of any disease symptoms. Samples were collected and labelled when symptoms were observed. GPS readings for each site were taken to aid in the development of an accurate distribution map. For Maui and Kauai, extension agents were contacted for help and they looked for the presence of disease symptoms in *Syngonium* grown on these islands. No symptomatic plants were found on Maui and Kauai.

Isolation of Fungi and Maintenance of Cultures

Collected diseased samples were brought to the lab and photographed. Diseased samples were processed to isolate potential pathogens from them. Two methods were used for isolation. Samples suspected to be infected with *C. fimbriata* were processed using both methods. Symptomatic areas of the samples were cleaned, washed, dissected into smaller pieces, surface sterilized with 5% or 10% Clorox and plated on water agar or incubated in a moist chamber. Moist chambers were prepared by placing a sterile filter paper moistened with a few drops of sterile distilled water on the bottom of a petri plate. Single hyphal tips of the mycelium growing out of the infected tissue samples on water agar were cut and transferred to 10% vegetable juice (V8) agar for growth and spore formation. 10% vegetable juice agar (V8) was prepared by dissolving 5.5 g USBC agar, 50 ml V8 juice (prepared by mixing 160 ml V8 with 0.384 g calcium carbonate) in 440 ml distilled water. In addition, samples incubated in moist chambers were observed for the formation of perithecia. When perithecia were observed, cream colored droplets of ascospores on the tips of perithecia were collected with a sterile needle and transferred to 10% V8 Agar. To obtain single ascospore pure cultures, ascospores were collected from cultures and streaked on water agar using a bacterial loop. After 24 hours, single germinating spores were cut using a sterile scalpel and transferred to V8 agar to establish the single spore cultures. Pure cultures were given unique identification numbers and were stored on V8 agar slants and in sterile water.

Identification

Cultures other than *C. fimbriata* were identified to genus based on morphological characteristics, including fruiting bodies, hyphae, conidiophores and spores (Barnett and Hunter, 2006; Uchida and Aragaki, 1979; Webster and Butler 1967). For *C. fimbriata*, morphological identification was confirmed by sequencing the ITS region. DNA was extracted using the DNeasy Plant Minikit (Qiagen, MD, USA) following manufacturer's instructions. ITS rRNA region was amplified using ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3 (White et al. 1990). PCR conditions were initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 3 min. PCR products thus obtained were electrophoresed in 1.5% agarose gel for 80 minutes at 100 volt and visualized under UV light. For purification, 5µl of PCR product was mixed with 2 µl of ExoSap-IT (Thermofisher Scientific, Waltham, MA) and incubated at 37°C for 15 minutes followed by incubation at 80°C for 15 minutes. 1 µl each of forward and reverse primers were added to 2.5 µl of the cleaned PCR Product in two tubes; diluted with Sterile water to make final volume of 15 µl. The PCR product thus processed was sequenced at both directions at Genewize Inc (La Jolla, California). The forward and reverse sequences obtained after sequencing were aligned using Geneious version 10.2.3 and manually edited to obtain the consensus sequences. Identity of the sequences were established by comparing the consensus sequences to the sequences in the NCBI GeneBank using BLASTn tool. Selected *Fusarium* species isolated were identified to species level by growing them on carnation leaf agar (Leslie and Summerell, 2007) and studying morphological characteristics including shape and size of macro and microconidia, presence or absence of false heads and/or chains and chlamydospores, and pigmentation as described by Leslie and Summerell, 2007.

Results

Survey for *Ceratocystis fimbriata* on *Syngonium*

Among the 22 places visited on Oahu (Figure 1), *Syngonium* was not found at HR Nursery (Waimanalo, Oahu) and Waimanalo Research Station (Waimanalo, Oahu), and *Syngonium* plants at Alluvian Nursery (Haleiwa, Oahu) and Ho'omaluhia Botanical Garden (Kaneohe, Oahu) were free of disease. At Hawaiian Sunshine Nursery (Waimanalo, Oahu), we were unable to obtain permission to collect samples. Of the 12 nurseries surveyed, only 4 nurseries grew *Syngonium* for commercial use. At the remaining locations, *Syngonium* was growing around fences as a landscape plant. The most common symptom observed was the darkened, soft, rotting tips of the aerial roots. Rotting of the roots of potted plants and darkened nodes of the plants were observed a few times. Samples were collected from *S. dodsonianum*, *S. schottianum, S. saggitatum*, *S. steyermarkii, S. wedlandii*, *S. auritum,* and a few unknown species of *Syngonium* and cultivars of *S. podophyllum* such as, "White butterfly", "Mango Allusion", "Neon", "Strawberry Cream", and "Golden". *Syngonium podophyllum* was the most common *Syngonium* species observed during the survey.

Samples were collected from two of the three nurseries surveyed in the Island of Hawaii. Plants at the third nursery were free of disease symptoms. Overall, no symptoms were observed on the foliage of the plants except for some wilting of a few leaves. Black rotting of the basal node and aerial roots and elongated lesions on the stem were observed upon closer examination. Black colored bodies resembling perithecia were observed in some symptomatic tissue with a hand lens at 10x magnification.

Extensions agents contacted for help on Maui and Kauai found no *Syngonium* plants with typical *C. fimbriata* symptoms. Thus, no samples were received from Maui and Kauai.

Figure 1. GPS points of the locations surveyed on Oahu.

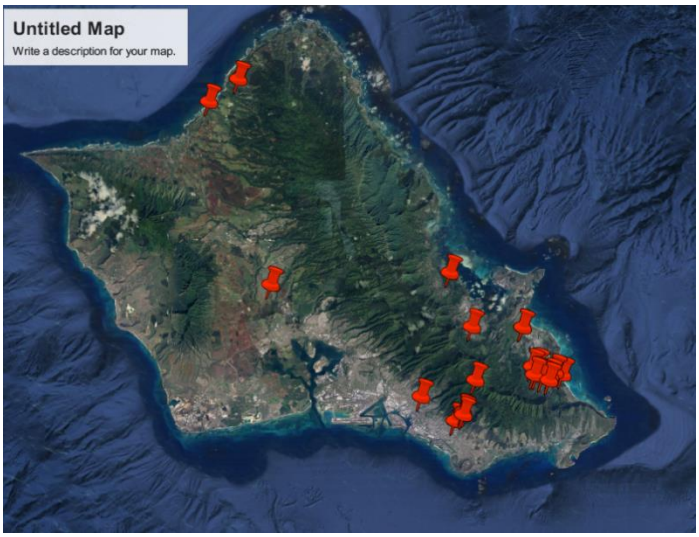


Figure 2. Symptoms of *Ceratocystis fimbriata* on *S. podophyllum* plants.

2A and 2B: Black rotting of the base and aerial roots of *S. podophyllum* plants. 2C: Brown internal discoloration immediately below the rotting node.

Isolation

Multiple fungi were isolated from the samples showing darkening, softening and rotting of the aerial root tips. In many cases, multiple fungi were isolated from a single sample. *Fusarium* was most commonly isolated. *Colletotrichum* species, *Actinopelte, Pestalotiopsis, Curvularia, Gliocladium*, *Phoma, Rhizoctonia solani*-like, *Oedocephalum*-like, *Drechslera*-like, *Stilbum*-like, and *Torula*-like fungi were isolated from samples collected on Oahu. Samples collected from two locations on Oahu showing symptoms of black node were suspected to be infected with *C. fimbriata*. For these samples, isolations were attempted using both water agar and the moist chamber method. *C. fimbriata* was not recovered with either of the methods. *Colletotrichum* and *Fusarium* species were isolated in both cases from the samples with black node symptoms.

Samples collected from Island of Hawaii were highly suspected to be infected with *C. fimbriata* due to the presence of perithecia observed with a hand lens. Isolations were attempted using both methods. *Ceratocystis fimbriata* was not recovered from samples plated on water agar, but from samples incubated in a moist chamber. Dark, flask-shaped perithecia containing cream-colored droplets of ascospores were observed after 3-4 days of incubation. Ascospore droplets were transferred to V8 plates with a sterile needle.

Figure 3. Black colored perithecia containing cream colored drops of ascospores on the surface of an infected *Syngonium* sample incubated in a moist chamber.

Table 1. Survey locations and *Ceratocystis fimbriata* isolated from Island of Hawaii.

ID	Collection location	ID	Collection location
3401	Novelty Greens, Hilo	3443	H. Eunice Nursery, Hilo
3404	Novelty Greens, Hilo	3445	H. Eunice Nursery, Hilo
3408	Novelty Greens, Hilo	3448	H. Eunice Nursery, Hilo
3410	Novelty Greens, Hilo	3452	H. Eunice Nursery, Hilo
3414	Novelty Greens, Hilo	3454	H. Eunice Nursery, Hilo
3416	Novelty Greens, Hilo	3458	H. Eunice Nursery, Hilo
3421	Novelty Greens, Hilo	3460	H. Eunice Nursery, Hilo
3423	Novelty Greens, Hilo	3465	H. Eunice Nursery, Hilo
3424	Novelty Greens, Hilo	3466	H. Eunice Nursery, Hilo
3428	Novelty Greens, Hilo	3470	H. Eunice Nursery, Hilo
3432	Novelty Greens, Hilo	3473	H. Eunice Nursery, Hilo
3433	Novelty Greens, Hilo	3475	H. Eunice Nursery, Hilo
3436	Novelty Greens, Hilo	3479	H. Eunice Nursery, Hilo
3440	H. Eunice Nursery, Hilo	3482	H. Eunice Nursery, Hilo

Table 2. Survey locations and *Fusarium* species isolated from the Islands of Oahu and Hawaii.

***Fusarium* species**	**Surveyed locations**
Fusarium solani	Koba's Nursery, Lyon Arboretum, Waimea Valley, Koolau farmers
Fusarium dimerum	Ultimate Innovations
F. decemcellulare	Ultimate Innovations, Lyon Arboretum
F. semitectum	Olomana Tropicals, Contemporary Landscaping
F. latericum	Olomana Tropicals
F. equiseti	Olomana Tropicals, Contemporary Landscaping, St. John Courtyard at the UHM, Foster Garden
F.guttiforme	Plant Hawaii
F. camptoceras	Plant Hawaii, Foster Garden
F. polyphialidicum	Plant Hawaii
F. oxysporum	Lyon Arboretum, Waimea Valley
F. heterosporum	New Mililani Nursery, Waimea Valley
Fusarium scirpi	Foster Garden
Fusarium spp	Contemporary Landscaping, Plant Hawaii, Foster Garden, Lyon Arboretum, Waimea Valley, Pang's Nursery, St. John Courtyard at UHM, Linchon Hall at EWC at UHM, City Mill, Down to Earth, Waihale Products, Hale Kuahine at EWC

Table 3. Survey locations and additional fungi isolated from the Islands of Oahu and Hawaii.

Other fungi	**Locations surveyed**
Colletotrichum spp	New Mililani Nursery[1], Sharon's Plant, Contemporary Landscaping, Plant Hawaii, Waimea Valley, Pang's Nursery, Linchon Hall at UHM, Koba's Nursery
Actinopelte sp	Olomana Tropicals, Plant Hawaii, Waimea Valley
Pestalotiopsis sp	Olomana Tropicals, Plant Hawaii, Lyon Arboretum, Waimea Valley
Curvularia sp	Plant Hawaii
Gliocladium sp	Lyon Arboretum
Stemphylium sp	Alluvian Nursery[1], City Mill[1]
Rhizoctonia solani - like	Plant Hawaii
Phoma spp	Ultimate Innovations[1], Olomana Tropicals, Contemporary Landscaping, St. John Courtyard at UHM, City Mill[1], Down to Earth
Oedocephalum - like	Koolau Farmers[1]
Drechslera - like	Alluvian Nursery[1]
Stilbum – like	H. Eunice[1], Novelty Greens[1]
Torula – like	Down to Earth
Ceratocystis fimbriata	H. Eunice[1], Novelty Greens[1]

1. At some commercial nurseries and retail garden stores potted *Syngonium* plants for sale were surveyed and at other locations landscape plants were surveyed.

Identification

Identification of the fungi to genus level was done by examination of the morphological characteristics based on published literature (Barnett and Hunter, 2006, Uchida and Aragaki 1979; Webster and Butler, 1967). Mycelium color, growth rate, conidiophores, formation of spores in conidiophores, type of fruiting bodies when present, shape and size of spores were documented. Eighty-two single spore isolates (multiple single spore isolates from a culture established by transferring ascospore droplet) were recovered from the samples collected from two locations in Hilo and twenty-eight isolates (one single spore isolate from each culture) were selected for further study (Table 1).

Suspected *Ceratocystis fimbriata* isolates were initially as white and slowly turned dark olive green on 10% V8 agar. Both sexual and asexual spores were observed. Cultures produced hyaline, cylindrical and doliform endoconidia in the phialides. Doliform endoconidia were produced and released in chains often embedded at the sides of perithecia. Dark colored perithecia with a globose base and long neck started forming in 10% V8 agar at approximately 5 days. Perithecia released cream-colored droplets of mucilage in which hat-shaped ascospores were embedded. Hat-shaped ascospores are a typical characteristic of *C. fimbriata*. Based on these morphological characteristics the fungus was identified as *C. fimbriata*. Detailed morphological and cultural characteristics are discussed in Chapter 4.

Morphological identification was confirmed by molecular analysis. All 15 isolates of *C. fimbriata* were sequenced and all had identical sequences for the ITS region. Nucleotide blast of the ITS sequences showed that all *C. fimbriata* collected during this study were 100% identical with 100% query coverage to *C. fimbriata* isolates previously collected from Hawaii by Dr. Janice Uchida (Accession Number: KU043248.1).

Discussion and Conclusion

Ceratocystis fimbriata was not isolated from any *Syngonium* samples collected from the island of Oahu. On Oahu, symptoms typical to *C. fimbriata* on *Syngonium* were difficult to find. Many species of *Fusarium*, *Colletotrichum* spp, *Actinopelte* sp, *Pestaloliopsis* sp, *Gliocladium* sp, *Phoma* spp, *Curvularia* sp, *Rhizoctonia solani* like fungus, *Oedocephalum* like fungus, *Drechslera* like fungus, *Stilbum* like fungus and *Torula* like fungus were isolated from the samples collected from Oahu. Eighty-two single spore *C. fimbriata* isolates (multiple single spore isolates from a culture obtained by transferring a ascospore droplet from perithecia that grew on the infected samples on moist chamber) were recovered from the samples collected from Novelty Greens, Hilo and H. Eunice Nursery, Hilo. *Ceratocystis*

fimbriata was recovered from 65% of the samples collected from Hilo. *Fusarium* and *Colletotrichum* were recovered from samples plated on water agar.

It was strange that *C. fimbriata* was not isolated from the infected samples plated on water agar. This might be because samples collected from island of Hawaii were surface disinfected with 5% Clorox instead of 10% Clorox regularly used in the Uchida lab. This might have caused insufficient surface sterilization leaving behind the saprophytes in the surface of the samples. Also, *C. fimbriata* is a slow growing pathogen compared to saprophytes like *Fusarium* species. It is possible that saprophytes grew faster and took over the whole plates before *C. fimbriata* could grow out of infected samples. The two nurseries in the island of Hawaii where *Syngonium* plants were found infected with *C. fimbriata* were in Hilo, not very far from each other and owned by relatives. The same population of *C. fimbriata* might have moved in planting materials.

CHAPTER 3

PATHOGENICITY TESTS OF *CERATOCYSTIS FIMBRIATA* ISOLATES COLLECTED FROM *SYNGONIUM* ON *SYNGONIUM* AND OHIA

Introduction

Ceratocystis was known to be present on sweet potato (Thorpe et al. 2005), *Syngonium* spp. and taro (*Colocasia spp*) (Thorpe et al. 2005; Uchida and Aragaki 1979) in Hawaii before Rapid Ohia Death was identified to be caused by two fungi in the genus *Ceratocystis*. *Ceratocystis fimbriata* from *Syngonium* is closely related to *C. lukuohia* infecting ohia trees by phylogenetic analysis (Barnes et. al 2018). *Ceratocystis fimbriata* from *Syngonium* is understudied and information on the genetic diversity of the pathogen in Hawaii is lacking. One of the objectives of this study was to test the pathogenicity of *C. fimbriata* isolates collected from *Syngonium* on ohia. *Ceratocystis fimbriata* isolates collected from *Syngonium* (Chapter 1) were tested for pathogenicity on *Syngonium* and ohia.

Materials and Methods

Plant Propagation

Syngonium plants were propagated by taking cuttings from *Syngonium* plants growing on campus and were tentatively identified as *S. podophyllum* cv. Mango Allusion based on the web resource (https://davesgarden.com). Cuttings were taken from healthy and vigorously growing *Syngonium* plants. *Syngonium schottianum, S. wedlandii, S. dodsonianum, S. podophyllum* cv. White Butterfly, cv. Neon, cv. Golden, and cv. Strawberry Cream were propagated from potted plants obtained from nurseries for

disease isolation. New growth having one or two nodes were taken; leaves and roots were removed. All the dead and decaying material on the new cutting was removed by trimming and washing the cuttings in soapy water. Cuttings were surface sterilized in 10% Clorox for approximately ten minutes. For rooting, cuttings were planted in a blend of sunshine blend #4, a peat mix. When rooted, cuttings formed new roots and leaves and they were transplanted individually into six-inch pots. Slow release fertilizer (10: 10: 10 NPK) was added to the surface of the potting mixture. Thrips were controlled by spraying the insecticide Brayer (B-cyfluthrin 0.0015% and Imidacloprid 0.0120% active ingredients) following the manufacturer's instructions. Plants were maintained at the Magoon greenhouse until they were used for pathogenicity tests.

Ohia plants were grown from seeds or obtained from a Hui Ku Maoli Ola Native Plant Nursery at Kaneohe, Oahu. Ohia seedlings in the greenhouse about 1 year old were transplanted to new pots and fertilized. Ohia plants used for inoculation were ~10-20 cm tall having ~ 2 cm stem circumference, grown in 4-inch pots. Before inoculation, ohia plants were maintained in a glass house with daily watering.

Experimental Design

Experiments were conducted in a completely randomized design. For ohia inoculations, four treatments were used, and each treatment was replicated twice. The experiment was conducted twice. The treatments are listed as follows:

Treatment 1: ohia seedlings were wounded by drilling into the main stem. Plants were inoculated with a spore suspension and wounds covered with parafilm.

Treatment 2: ohia seedlings were wounded by drilling into the main stem. Plants were inoculated with a spore suspension and wounds not covered with parafilm.

Treatment 3: ohia seedlings were wounded by drilling into the mainstream. Plants were inoculated with sterile distilled water and wounds covered with parafilm.

Treatment 4: ohia seedlings were wounded by drilling into the mainstream, Plants were inoculated with sterile distilled water and wounds not covered with parafilm.

For *Syngonium* inoculations, two treatments were used and listed as follows:

Treatment 1: *Syngonium* plants were non-wounded and thoroughly sprayed with a spore suspension.

Treatment 2: *Syngonium* plants were non-wounded and thoroughly sprayed with sterile distilled water. Plants sprayed with sterile distilled water served as the control group. Each treatment was replicated twice. This experiment was conducted twice with three replications per treatment.

A third experiment was conducted to determine the response of different cultivars of *S. podophyllum* to *C. fimbriata.* A single-spore isolate was used (*C. fimbriata* 3459). *Syngonium podophyllum* cvs. Mango Allusion, Neon, Strawberry Cream, and two unknown cultivars (*S. podophyllum* cultivar 1 and *S. podophyllum* cultivar 2) were used. Six plants of each cultivar (3 inoculated and 3 controls) were used except for *S. podophyllum* cv. Strawberry Cream and *S. podophyllum* cultivar 2 for which 4 plants were used (2 inoculated and two controls). The procedure for inoculum preparation and inoculation was the same as described below for *Syngonium*. A 0-5 disease scale was developed to score the plants for disease incidence and severity. Plants were scored 9 days post-inoculation. The scale for scoring inoculated *Syngonium* plants was as follows:

0: No disease

1: A few light gray spots on the leaves and petioles.

2: Dark spots on the leaves and black elongated lesions on the petiole, sometimes coalescing to form bigger lesions, 3 – 4 cm in diameter.

3: Coalesced lesions on the leaves, black rotting of the nodes and yellowing and wilting of outer leaves.

4: Coalesced lesions on the leaves, black rotting of the nodes and death of outer leaves.

5: Wilting and death of whole plant.

Inoculum Preparation

For inoculations, *C. fimbriata* isolates 3421 and 3459 were grown on 10% V8 agar under continuous florescent light for three days at room temperature (~25°C). Spores were collected from actively growing cultures using a sterile titanium loop and streaked onto fresh 10% V8 plates. This resulted in abundant growth and high concentrations of spores. Spores were harvested on the third day with collection of cylindrical endoconidia only since ascospores of *C. fimbriata* started to form on 10% V8 agar on day 5. Endoconidia were collected by pouring ~3 ml of sterile distilled water on culture plates and dislodging spores with a rubber spatula. The spore suspension from the first plate was transferred to three more fresh plates and dislodging was repeated with a rubber spatula to prevent the dilution of spores. Spore suspensions collected from the four plates were poured into a beaker and filtered with Kimwipe. Endoconidia concentration was determined by counting a subsample of spore using a hemocytometer and spore concentration was adjusted by diluting the suspension with sterile distilled water. For inoculation of ohia plants, spore suspension was adjusted to $1x10^7$ spores per ml of sterile distilled water while for inoculation of *Syngonium* plants spore concentration was adjusted to $1x10^6$ spores per ml of sterile distilled water.

Inoculation

Syngonium plants were sprayed to drip with a spore suspension adjusted to $1x10^6$ spores per ml in sterile distilled water. Control *Syngonium* plants were sprayed with sterile distilled water. All inoculated

plants were incubated under 100% relative humidity for 24 hours in plastic bags. After 24 hours, plants were removed from plastic bags and kept under laboratory conditions.

Slanting holes were drilled into the primary stem of the ohia seedlings with a hand drill (1/16 of an inch in diameter). Plants were drilled approximately half way thorough the stem. Twenty µl of the spore suspension adjusted to $1x10^7$ spores per ml in sterile distilled water was pipetted into the drilled holes using 20µl pipette tips. Twenty µl of sterile distilled water was pipetted into the control plants. Plants were incubated at 100% humidity in plastic bags for 24 hrs, then removed from the bags the following day. Plants were maintained in the laboratory next to a large window at 25°C and monitored daily. The wounds were either covered with parafilm or left uncovered. Each treatment had two plants.

Observation for Symptoms and Reisolation

Inoculated *Syngonium* plants were observed daily for disease development. If disease was observed, symptoms were recorded, and plants were photographed. Reisolation from symptomatic plant parts was performed 14 days post-inoculation and reisolation frequency was recorded. Reisolated cultures were compared with the original culture to confirm they are identical using the morphological characteristics including sexual and asexual spores and sexual fruiting body (perithecia).

Results

Inoculation

Symptoms were observed on *Syngonium* plants inoculated with *C. fimbriata* three days post-inoculation (DPI) (Figure 4). After 3 DPI, a few light gray, water soaked spots sometimes surrounded by yellow halos were observed on leaves and light gray dots were observed on the petiole. The lesions on the

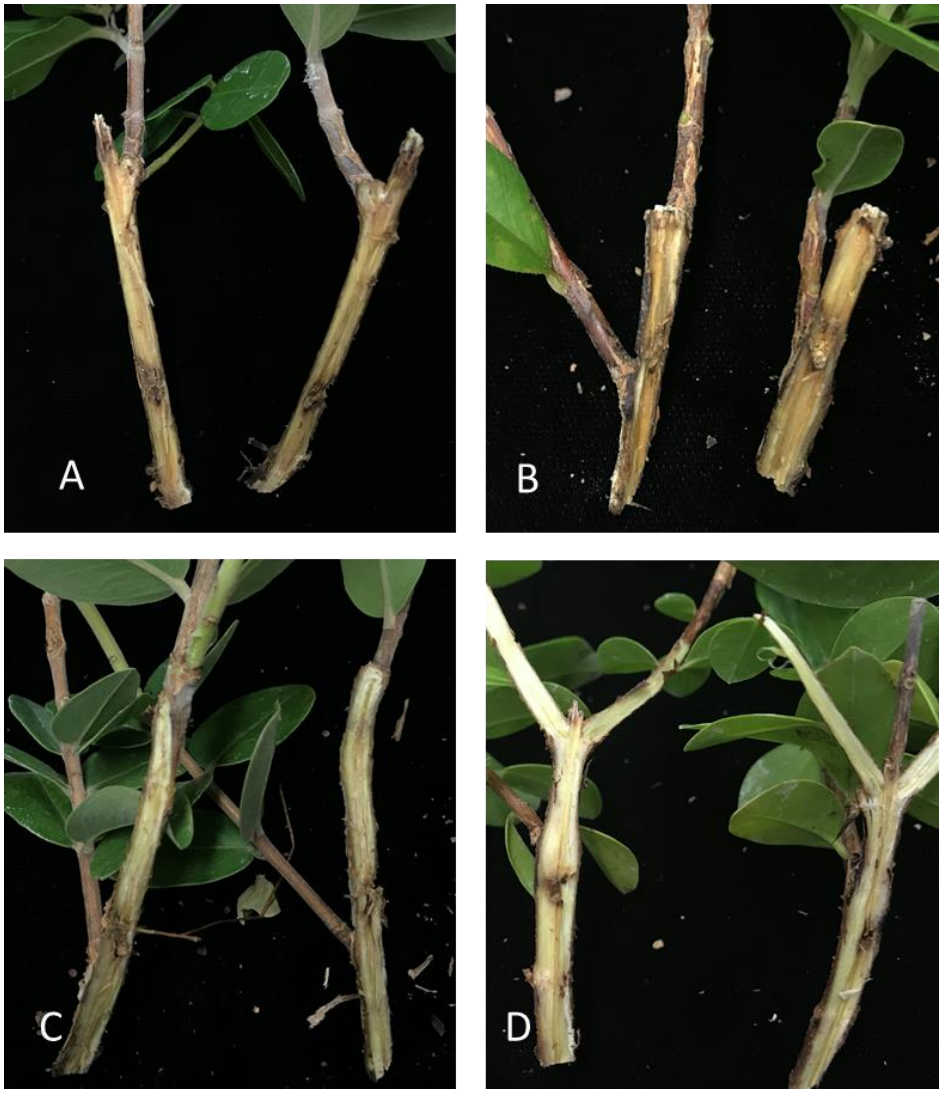
leaves continued to expand in size and became 1-2 cm wide after 7 days. The lesions on the leaves sometimes coalesced together to form dark patches of infected tissues. The lesions on the petiole became dark and elongated. Darkening of the nodes and black rotting of the nodes and aerial roots were observed on 7th day, as the disease continued to progress. Outer leaves wilted, most leaves died 14 days post-inoculation. The experiment was repeated once with similar results. No control plants showed disease symptoms.

No symptoms were observed on inoculated ohia seedlings even three months post-inoculation. There were no differences observed between control and inoculated seedlings. Seedlings formed new leaves. About 1-month post-inoculation, one of the ohia seedling inoculated with isolate 3459 and covered with parafilm and one wounded control seedling covered with parafilm wilted and died. The inoculated plant was dissected, and the pith of the stem was dead and discolored. Tissue at the point of inoculation, below the point of inoculation and above the inoculation was surface sterilized with 10% Clorox and plated on water Agar. Single hyphae of the mycelium growing out of the sample was cut and transferred to 10% V8 agar plates. 10% Clorox properly sterilized the surface and *C. fimbriata* grew out of infected *Syngonium* plants. Thus, same protocol was followed for ohia as well.

Figure 4. Symptoms on *Syngonium* spray inoculated with *Ceratocystis fimbriata* isolate 3401 on S. podophyllum cv. Mango Allusion.

4A: Healthy *Syngonium* plant before inoculation, 4B: 3 days post-inoculation, 4C: 7 days post-inoculation, 4D: 14 days post inoculation. 4E: dark elongated lesions on petiole, 4F: Black rotting of nodes and aerial roots.

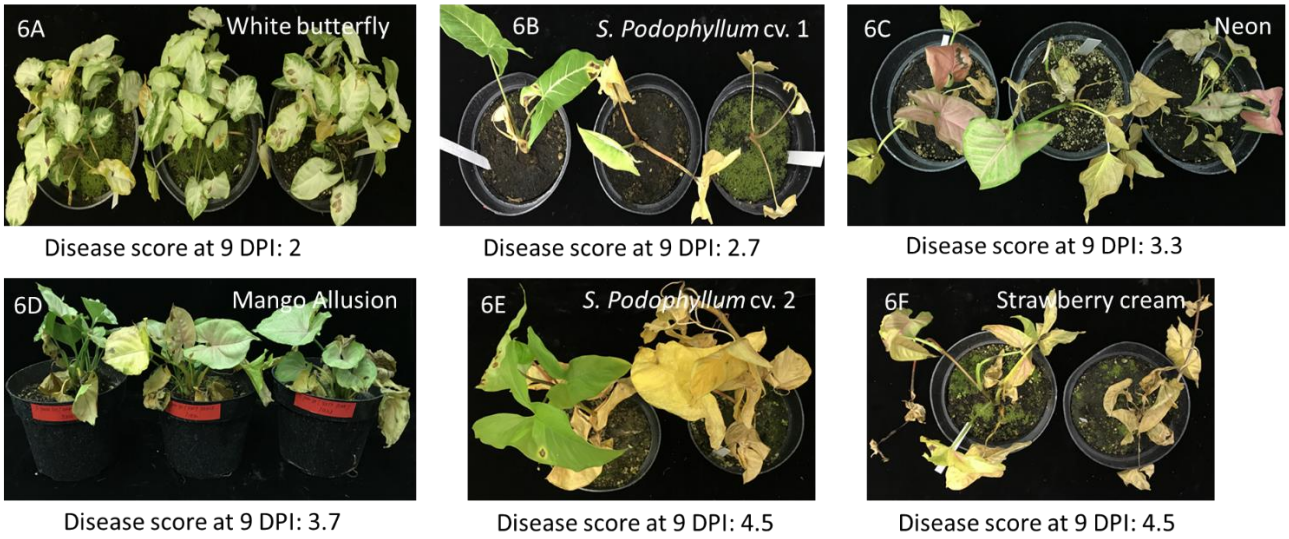
Figure 5. Symptoms of *C. fimbriata* inoculated ohia seedlings dissected 106 days post-inoculation.



A: Ohia plants inoculated with isolate 3401 and wound left uncovered, B: Inoculated with isolate 3401 and wound covered with parafilm, C: Inoculated with isolate 3459 and wound left uncovered, D: Inoculated with isolate 3459 and wound covered with parafilm.

All of the cultivars of *S. podophyllum* tested were susceptible to *C. fimbriata*. By day 9 *S. podophyllum* cv. Strawberry Cream and *S. podophyllum* cv 2 were highly susceptible with overall disease scores of 4.5. *S. podophyllum* cv. Mango Allusion and *S. podophyllum* cv. Neon were moderately- highly susceptible with scores of 3.7 and 3.3, respectively. *S. podophyllum* cv. White Butterfly and *S. podophyllum* cv. 1 were somewhat resistant with disease scores of 2 and 2.7, respectively. Disease continued to progress in all cultivars. Disease progressed most quickly in *S. podophyllum* cv. Strawberry Cream and *S. podophyllum* cv. 2 and symptom development was slowest in *S. podophyllum* cv. White Butterfly and *S. podophyllum* cv. 1. *Syngonium podophyllum* cv. Strawberry Cream was most susceptible and both plants died 14 days post-inoculation. Thus, *C. fimbriata* was reisolated from dead plants for *S. podophyllum* cv. Strawberry Cream. All control plants showed no symptoms.

Figure 6. Symptoms on *Syngonium podophyllum* cultivars inoculated with *C. fimbriata* isolate 3459 at 9 days post - inoculation.



6A: *S. podophyllum* cv White Butterfly, 6B: *S. podophyllum* cv 1, 6C: *S. podophyllum* cv Neon, 6D: *S. podophyllum* cv Mango Allusion, 6E: *S. podophyllum* cv 2, 6F: *S. podophyllum* cv Strawberry Cream.

Reisolation

Ceratocystis fimbriata was successfully reisolated from at least one of the *Syngonium* plants inoculated with isolates 3401 and 3459 for all experiments, including the cultivar screening. *C. fimbriata* was easily reisolated from leaf, petiole, and nodes with a 100% success rate of recovery; however, the reisolation frequency from roots was between 50-70%. The morphology of the cultures recovered after reisolation were identical to the original cultures used for inoculation, thus fulfilling Koch's postulates.

A *Pleiochaeta*-like fungus was isolated from the inoculated ohia seedling that died during the experiment while *Pestalotia* sp. and a *Phoma*-like fungus were reisolated from inoculated ohia plants that survived until the end of the experiment (including control plants). *Ceratocystis fimbriata* was not isolated from any ohia plant used in the experiment.

Discussion and Conclusion

Koch's postulate was established with *C. fimbriata* using multiple cultivars of *Syngonium*. The isolates tested were collected from *Syngonium* found at two nurseries located on Hawaii Island. Symptoms were observed on all aerial parts of the plant including leaf, petiole, node and aerial roots. No symptoms were observed on the roots below the soil surface. This is in contrast to *Syngonium* plants from which *C. fimbriata* was isolated where no leaf spots were observed. Leaf spots on *Syngonium* plants upon spraying with the conidial solution were also observed by Dr. Janice Uchida who reported the presence of *C. fimbriata* on *Syngonium* from Hawaii (Uchida and Aragaki 1979). *Ceratocystis fimbriata* is commonly referred to as a wound pathogen and requires a wound to get into its woody hosts (Barnes et al. 2003; Kile 1993; Roux et al. 2004; Van Wyk et al. 2005). However, with *Syngonium*, infection occurred in non-wounded plants.

One of the most important objectives of this project was to determine the distribution of *C. fimbriata* from *Syngonium* state-wide and test the pathogenicity of the collected isolates on ohia. All inoculated ohia seedlings used in the experiment except two continued to remain healthy after being wounded and inoculated with *C. fimbriata* isolates 3401 and 3459 throughout the length of the experiment. Similar findings were recently reported by (Barnes et al. 2018). In this study, optimum conditions for the infection to occur were provided by wounding the plants to expose the vascular bundles. One set of plants were also wrapped with parafilm following inoculation so that the inoculum would not dry out. Barnes et al. (2018) identified pathogens killing ohia trees as two new species in the *C. fimbriata* species complex. *Ceratocystis lukuohia* and *C. huliohia* were identified based on morphological studies, phylogenetic analysis, intersterility tests and pathogenicity tests. *Ceratocystis lukuohia* infecting ohia is genetically close to *C. fimbriata* from *Syngonium*, but different enough (genetically, morphologically and host range) to be a different species. Pairings of *Ceratocystis* isolates from these two hosts produced deformed ascospores with low germination capacity, further supporting the biological species concept (Barnes et al. 2018). *Ceratocystis* species tend to be specialized in the host that they infect. Cacao, sweet potato and sycamore isolates are uniquely infected the host they were isolated from (Baker et al. 2003). Aspen (*Populus tremuloides*) and Hickory (*Carya* spp) isolates also only infect the respective hosts (Johnson et al. 2005). *Ceratocystis fimbriata* isolates from *Syngonium* and *Xanthosoma* are more pathogenic on *Syngonium* as compared to *Colocasia* isolates and *Colocasia* isolates cause more petiole discoloration in *Colocasia* as compared to *Syngonium* isolates, while *Ceratocystis* isolates from woody hosts including *Mangifera* and *Ficus* are not pathogenic to the aroids (Thorpe, Harrington, and Uchida 2005). Similarly, *Xanthosoma* isolates were non pathogenic to plane, cacao, sweet potato and coffee (Baker et al. 2003).

Ceratocystis fimbriata was not isolated from the two ohia seedlings that died during the experiment. The cause of their death remains unknown, but it is highly unlikely that *C. fimbriata* killed the ohia seedlings. *Pestalotia* sp. isolated from these two seedlings is a commonly found plant endophyte that colonizes wounds under moist conditions (Maharachchikumbura et al. 2011) and might have contributed

to the seedling death. *Phoma* spp can be saprophytic or parasitic and causes leaf spots, cankers and *Pleiochaeta* is known to cause brown leaf spot (Gur and Frenkel 2016) and root rot of lupins (Luckett et al. 2009).

Reisolation of *C. fimbriata* from roots was difficult compared to other parts of *Syngonium* including the leaf, stem and petiole. Aerial roots of the *Syngonium* plants died more quickly than the thicker parts of the plants like petioles and stems. It is possible that the rotten *Syngonium* roots were colonized by saprophytes in the pot, making re-isolation of *C. fimbriata* unlikely.

No cultivar of *Syngonium podophyllum* used in this experiment showed resistance to *C. fimbriata*. 'White Butterfly', the most popular commercial cultivar, was more resistant compared to other cultivars; however, infection occurred, and the symptoms were enough to be aesthetically unappealing as an ornamental plant.

CHAPTER 4

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF *CERATOCYSTIS FIMBRIATA* ISOLATES FROM *SYNGONIUM*

Introduction

Ceratocystis fimbriata can easily be grown in artificial media. On V8 plates, *C. fimbriata* initially forms hyaline mycelium which becomes dark as the culture ages. Abundant cylindrical endoconidia are formed in the culture. Doliform endoconidia are not as abundant as cylindrical endoconidia although they are numerous. Aleurioconidia are thick walled spores which are formed at the later stage of development primarily after 10 days in the plates. *Ceratocystis fimbriata* also produces flask shaped structures with round bases and elongated necks called perithecia. Perithecia are the sexual fruiting structure inside which ascospores are produced and released in the form of a droplet of spores embedded in cream-colored mucilage. In culture, growth and sporulation is affected by temperature. Different strains of the fungus tend to grow better at certain temperatures. The objective of this study was to measure and compare morphological characters and growth rates of *C. fimbriata Syngonium* strains from Hawaii.

Materials and Methods

Morphological Characteristics

To study the cultural and morphological characteristics, *C. fimbriata* isolates 3401, 3459, 3466 and 3421 were grown on 10% V8 agar plates under continuous florescent light at approximately 25°C. Isolates 3401 and 3421 were collected from Novelty Greens and isolates 3459 and 3466 were collected from H. Eunice Nursery. Both nurseries are located on Hawaii Island. One hundred measurements were

taken for cylindrical endoconidia, ascospores and chlamydospores while 50 measurements were taken for perithecia and doliform endoconidia for each isolate. Length and width were measured for cylindrical endoconidia, doliform endoconindia and ascospores. Perithecia were measured for their neck length (including ostiolar hyphae), base length and base width. Base length for perithecia was the diameter of the base parallel to the neck of the perithecia and width of the base was the diameter measured at right angle to the neck of the perithecia. Measurements for cylindrical endoconidia and ascospores were taken from 5-7 days old culture, doliform endoconidia from 8-10 days old culture and chlamydospores from 14 days old culture. Small blocks of agar were cut from actively growing cultures and mounted on glass slides with cover slips and observed under a compound microscope. Cylindrical endoconidia, doliform endoconidia, ascospores and chlamydospores were measured under 600X while perithecia were measured under 100x magnification. Color, texture and other morphological features of the spores were noted. Images were captured using an Infinity 2 microscope camera mounted on an Olympus BX43 microscope.

Measurement of the Growth Rate

Growth rate of the three isolates of *C. fimbriata*, 3401, 3459 and 3466 were characterized. Isolates were grown on 10% V8 agar under continuous florescent light. Temperatures from 10˚C - 35˚C were tested at 5˚C increments. Circular plugs of the agar were taken from actively growing 4 days old cultures with the help of a 5 mm boring cork. Three plugs, one each from three isolates, were placed on 100 mm x 15 mm petri plates with 10%V8 agar. Three such plates were made so that each treatment (isolate) was replicated three times. Plates were incubated at the required temperatures (Thermofisher Scientific, Waltham, MA). Radial growth of the mycelium was measured at 3, 5, 7, 9 and 14 days.

Statistical Analysis

Statistical analysis was done using R-studio version 3.4.3 (R Core Team 2017). Normality of the data was checked with Shapiro-Wilk's test. Analysis of variance (ANOVA) was conducted at 0.05 level of significance to find if the growth rate for the isolates differed significantly at 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. When growth rates of isolates differed significantly at a particular temperature or when different temperatures had significant effects on the growth rate of an isolate, multiple comparison was done, and the groups were separated using LSD test. R package Agricolae (Felipe de Mendiburu 2017) was used for LSD test. P-value was adjusted by Holm's method for P-value adjustment.

Results

Measurement of Growth Rate

The growth of *C. fimbriata* isolates 3401, 3459 and 3466 did not occur at 10°C and 35°C. No measurable growth was observed at these temperatures. Temperature was significant while the isolate factor was insignificant in the analysis of variance (ANOVA) at 0.05 level of significance (Table 5). Within the parameters tested, results indicate that temperature was a significant factor determining the radial growth of all of the isolates while the radial growth did not significantly differ according to the isolates (Table 5). However, isolate 3466 had highest growth at 15°C (1.58 cm), 20°C (2.30 cm) and 25°C (2.43 cm). Similarly, isolate 3401grew the best at 30°C (1.86 cm). At 14 days, the largest radial growth was observed at 25°C for all three isolates (Figure 7). The radial growth was 2.41 cm, 2.41 cm and 2.43 cm for *C. fimbriata* isolates 3401, 3459 and 3466, respectively. Growth of the isolates increased as the temperature was increased until 25°C and then decreased (Figure 8).

Table 4. Mean radial growth (in mm) and standard error of mean (SEM) of *Ceratocystis fimbriata* isolates at different temperatures after 14 days of incubation.

Temperature/Isolates	3401	3459	3466
15°C	14.8 ± 0.33	13.5 ± 0.57	15.8 ± 1.20
20°C	21.5 ± 1.04	22.7 ± 0.88	23 .0 ± 1.00
25°C	24.2 ± 0.92	24.2 ± 0.92	24.3 ± 1.33
30°C	18.7 ± 0.33	18.3 ± 0.33	17.7 ± 0.33

Measurements are presented as Mean radial growth (mm) + SEM and each measurement is average of three measurements of three colonies.

Table 5. Analysis of variance (ANOVA) of radial growth of *Ceratocystis fimbriata* isolates 3401, 3459 and 3466 after 14 days of incubation at 15°C, 20°C, 25°C and 30°C at 0.05 significance.

	Df	Sum sq	Mean sq	F value	Pr(>F)
Isolate	2	1.9	0.97	0.459	0.636
Temperature	3	490.5	163.50	77.703	3.03e-14 ***
Residuals	30	63.1	2.10		

Df = Degree of freedom, Sum Sq= Sum of squares, Mean Sq= Mean sum of squares, ***= Highly significant.

Figure 7. Radial growth of *Ceratocystis fimbriata* isolates 3401, 3459 and 3466 in millimeters, at 15°C, 20°C, 25°C and 30°C after 14 days of incubation.

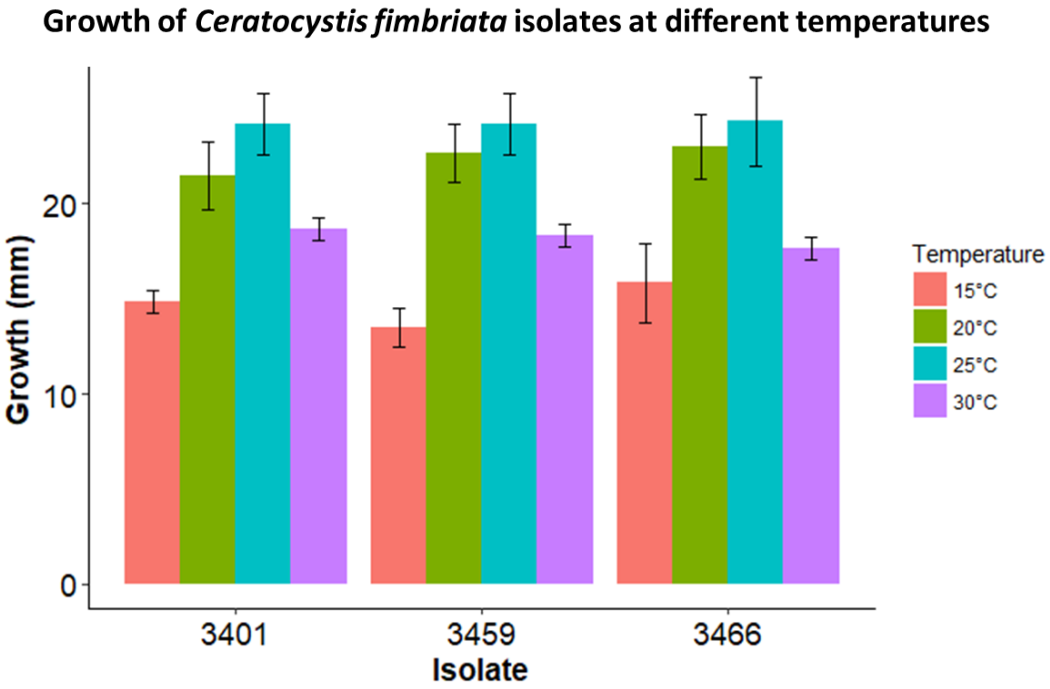
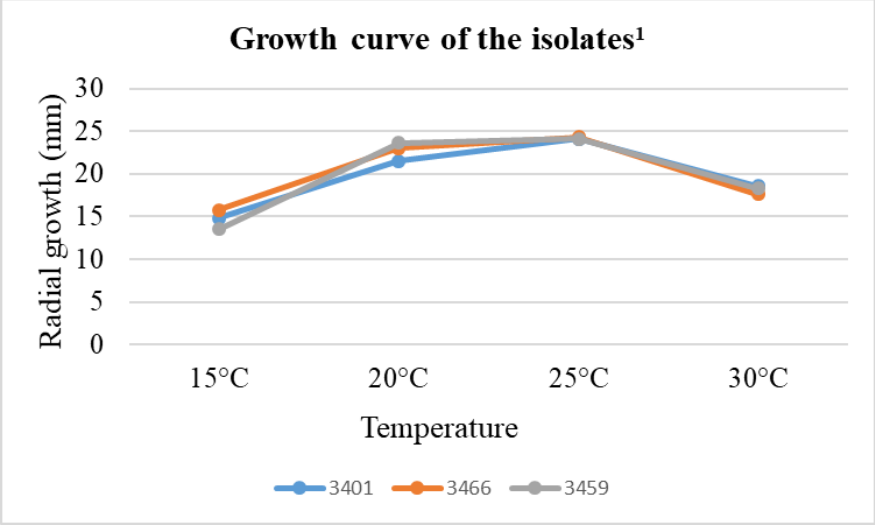


Figure 8. Growth curve of *Ceratocystis fimbriata* isolates 3401, 3459 and 3466 at 15°C, 20°C, 25°C and 30°C. Radial growth is measured after 14 days of incubation.



1. Each measurement is an average of three colonies.

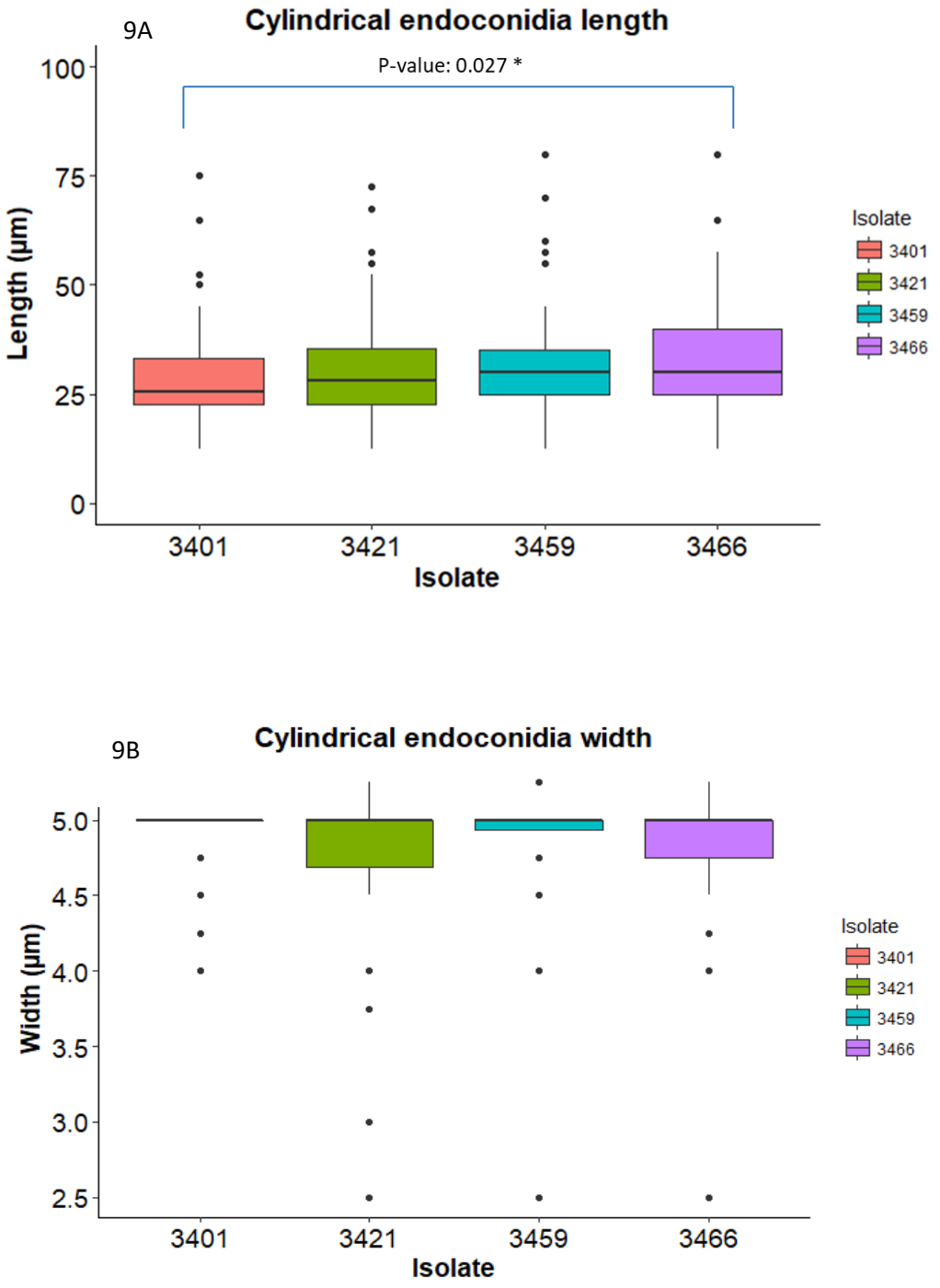
Cultural and Morphological Characteristics

Cylindrical endoconidia from *C. fimbriata* isolate 3401 were significantly longer compared to isolate 3466 (Figure 9A). The cylindrical endoconidial length among 3401, 3421 and 3459 was not different at 0.05 level of significance. All four isolates were similar in terms of cylindrical endoconidia width in statistical analysis. Similarly, ascospores of *C. fimbriata* isolate 3401 were significantly longer and wider compared to those of isolates 3459, 3466 and 3421 (Figure: 10A and 10B). Aleurioconidia length for all four isolates were not statistically different while the aleurioconidia width was highly variable among all four isolates. Pairwise comparison of the aleurioconidia width among isolates revealed significant difference among all pairwise comparisons except for the pairwise comparison between isolate 3401 and 3421. Doliform endoconidial length for isolates 3401 and 3421 were statistically similar, as were for isolates 3459 and 3466. However, doliform endoconidial length for isolates 3401 and 3421 were significantly different compared to isolates 3459 and 3466 (12A). Pairwise comparison of perithecial length gave significant p-values except for the pairwise comparison between isolates 3421 and 3466 (Figure 13A and 13B). Measurement of neck length of perithecia was subjected to pairwise comparison using Dunn test at 0.05 level of significance. Results revealed that the neck length of isolates 3459 and 3466 were not significantly different compared to each other, whereas all other pairwise comparison revealed significantly different neck length among isolates.

Table 6. Mean length and width (in µm) of cylindrical endoconidia with standard error of mean (SEM) of four *Ceratocystis fimbriata* isolates, 3401, 3421, 3459 and 3466.

Isolate	Cylindrical conidia length (mean ± SEM)	Cylindrical conidia width (mean ± SEM)
3401	28.7 ± 1.01	4.9 ± 0.024
3421	30.9 ±1.15	4.7 ± 0.05
3459	30.9 ± 1.12	4.8 ± 0.06
3466	33.1 ± 1.24	4.7 ± 0.06

Figure 9. Graphical representation of cylindrical endoconidial length and width as boxplots.

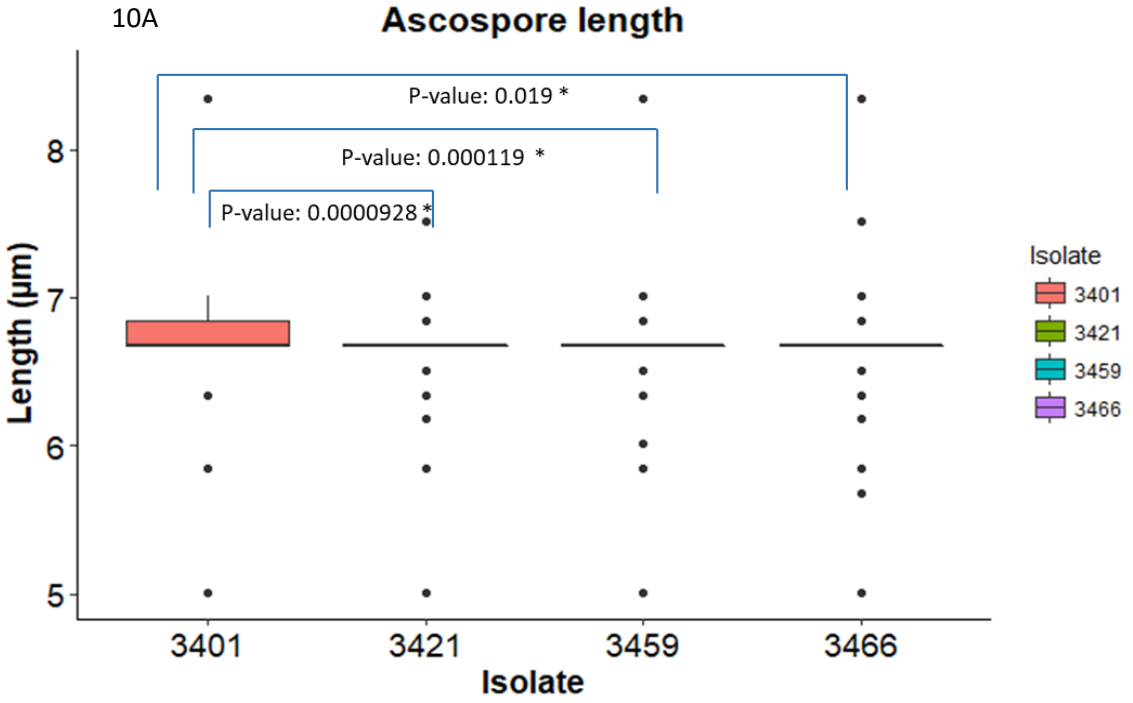


The bars on the box plots represent standard deviation. The black dots represent the outliers in the data set. The P-value was obtained by pairwise comparison of the dimension among isolates. The pairwise comparisons with significant P- values are significantly different from each other.

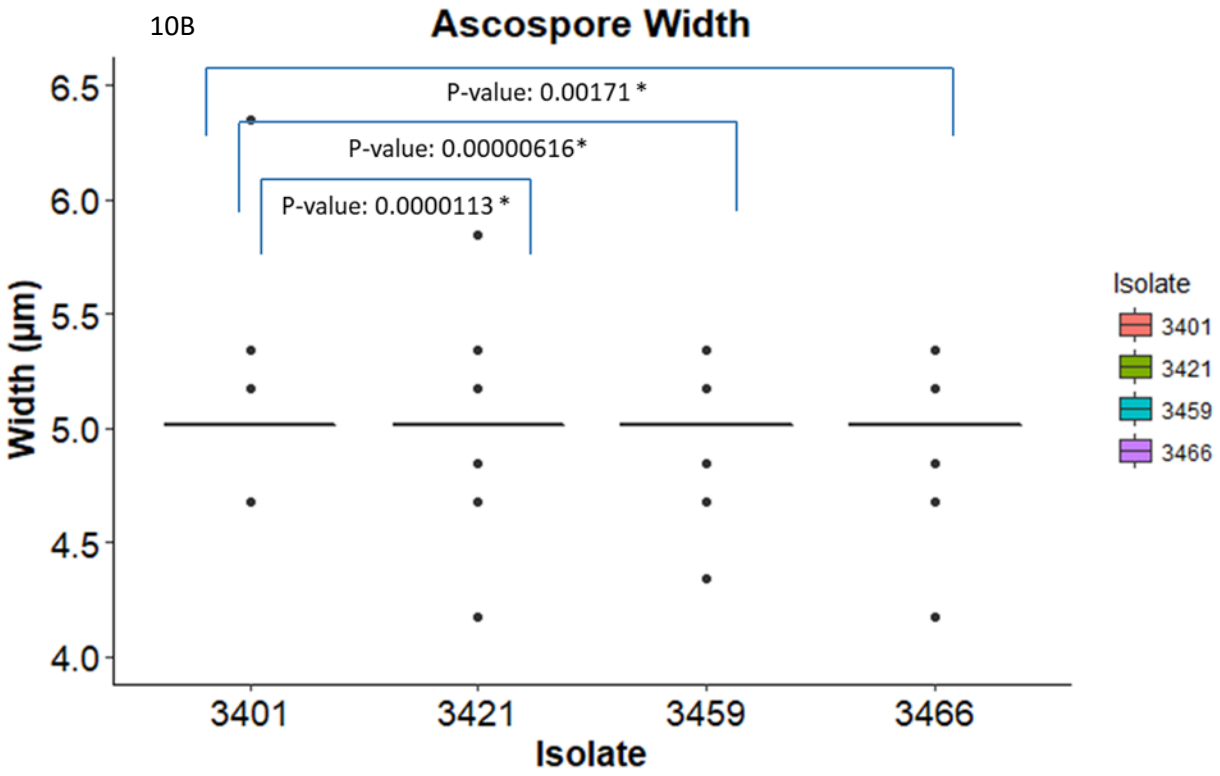
Table 7. Mean length and width (in µm) of ascospores with standard error of mean (SEM) of four *Ceratocystis fimbriata* isolates, 3401, 3421,3459 and 3466.

Isolate	Ascospore length (mean ± SEM)	Ascospore width (mean ± SEM)
3401	6.9 ± 0.708	5.3 ± 0.073
3421	6.6 ± 0.032	4.9 ± 0.04
3459	6.6 ± 0.050	4.9 ± 0.053
3466	6.7 ± 0.072	5.0 ± 0.061

10A



10B

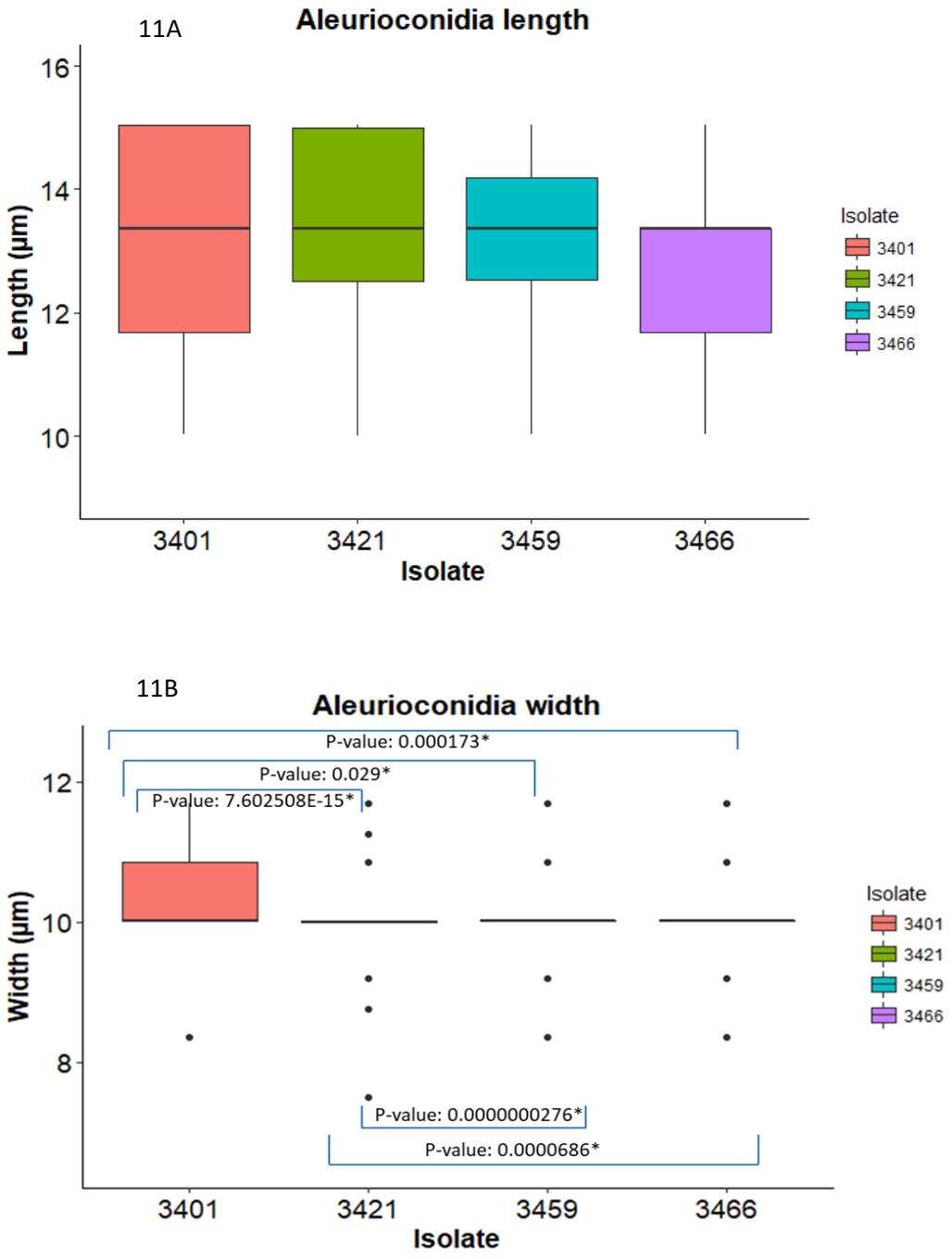


The bars on the box plots represent standard deviation. The black dots represent the outliers in the data set. The P-value was obtained by pairwise comparison of the dimension among isolates. The pairwise comparisons with significant P- values are significantly different from each other.

Table 8. Mean length and width (in µm) of aleurioconidia (chlamydospores) with standard error of mean (SEM) of four *Ceratocystis fimbriata* isolates, 3401, 3421,3459 and 3466.

Isolate	Aleurioconidia length (mean ± SEM)	Aleurioconidia width (mean ± SEM)
3401	13.4 ± 0.131	10.5 ± 0.076
3421	13.3 ± 0.144	9.9 ± 0.070
3459	13.3 ± 0.143	10.2 ± 0.064
3466	13.2 ± 0.138	10.0 ± 0.075

Figure 11. Graphical representation of aleurioconidia (chlamydospore) length and width as boxplots.

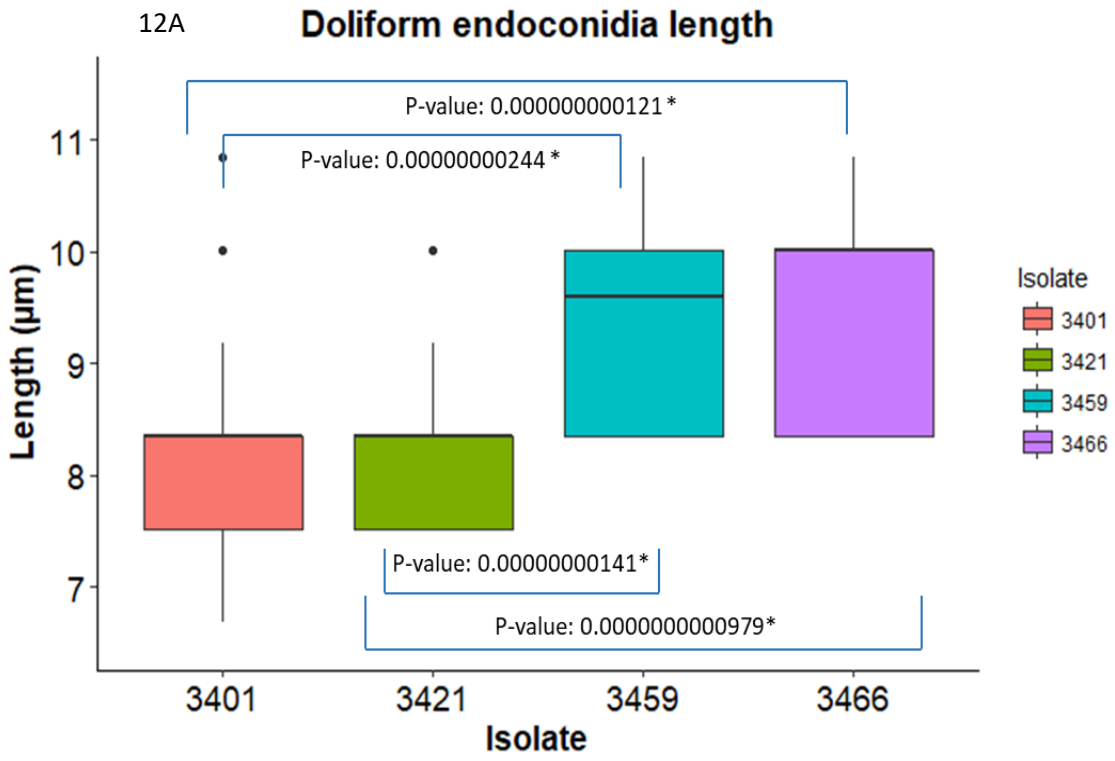


The bars on the box plots represent standard deviation. The black dots represent the outliers in the data set. The P-value was obtained by pairwise comparison of the dimension among isolates. The pairwise comparisons with significant P- values are significantly different from each other.

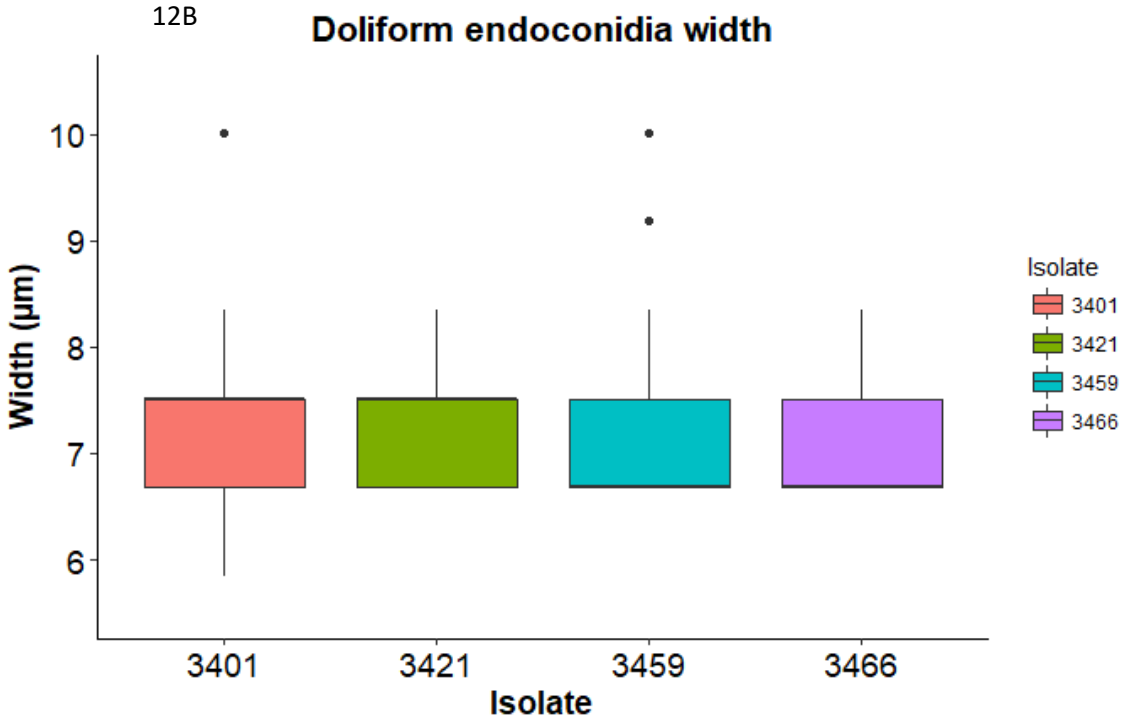
Table 9. Mean length and width (in µm) of doliform conidia with standard error of mean (SEM) of four *Ceratocystis fimbriata* isolates, 3401, 3421, 3459 and 3466.

Isolate	Doliform endoconidia length (mean ± SEM)	Doliform endoconidia width (mean ± SEM)
3401	8.2 ± 0.116	7.3 ± 0.112
3421	8.2 ± 0.093	7.4 ± 0.081
3459	9.4 ± 0.116	7.3 ± 0.112
3466	9.6 ± 0.126	7.1 ± 0.093

Figure 12. Graphical representation of doliform conidia length and width as boxplots. The bars on the box plots represent standard deviation.



12B

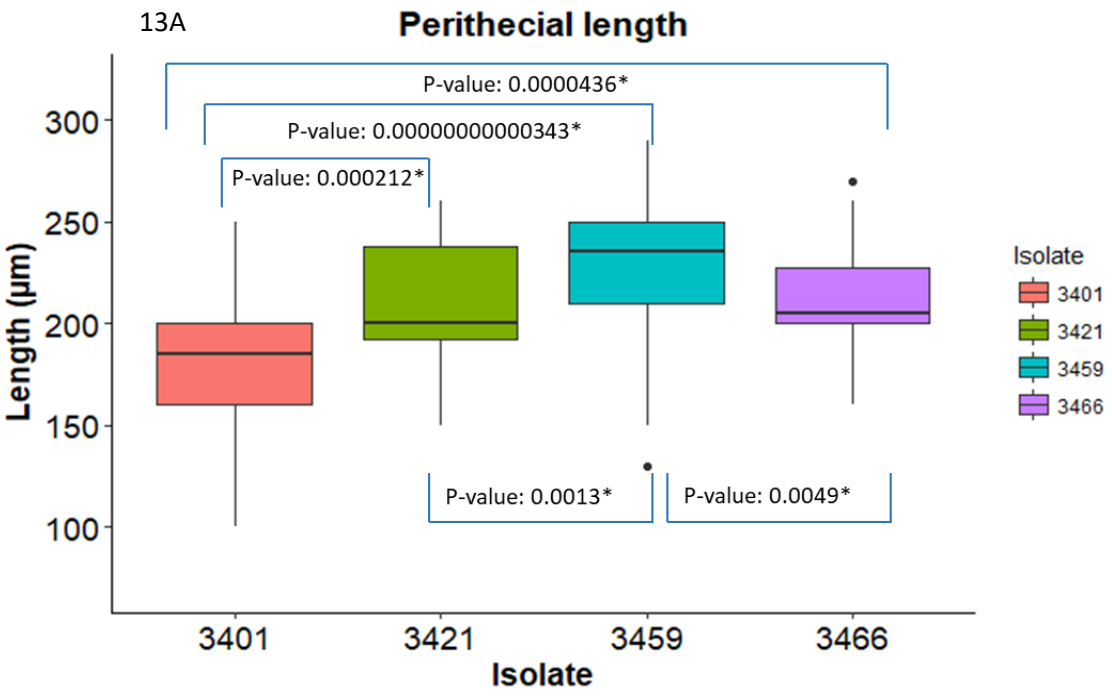


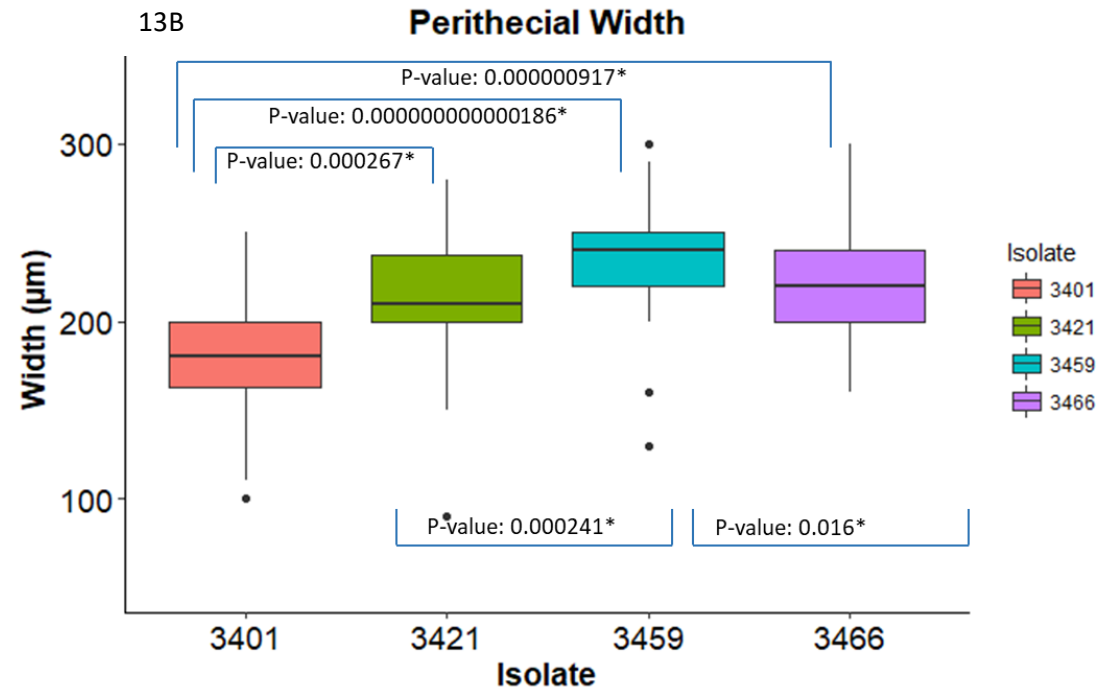
The bars on the box plots represent standard deviation. The black dots represent the outliers in the data set. The P-value was obtained by pairwise comparison of the dimension among isolates. The pairwise comparisons with significant P- values are significantly different from each other.

Table 10. Mean length, width and neck length (in µm) of perithecia with standard error of mean (SEM) of four *Ceratocystis fimbriata* isolates, 3401, 3421, 3459 and 3466.

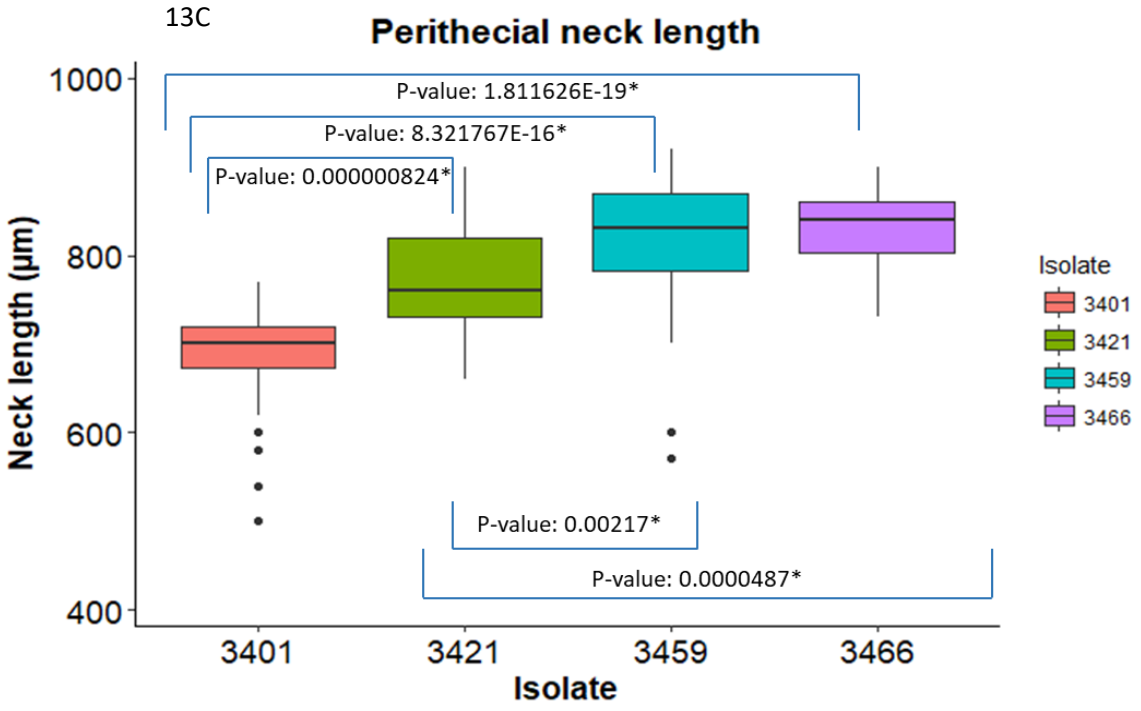
Isolate	Basal length (mean ± SEM)	Basal width (mean ± SEM)	Neck length (mean ± SEM)
3401	180 .0 ± 4.755	182.6 ± 4.996	629.6 ± 7.579
3421	208.8 ± 3.808	212.2 ± 4.465	774.8 ± 7.893
3459	229.2 ± 4.779	237.2 ± 4.840	815.2 ± 9.852
3466	212.0 ± 3.568	223.8 ± 4.207	834 ± 6.074

Figure 13. Graphical representation of perithecia basal length perithecia basal width and neck length of perithecia as boxplots. The bars on the box plots represent standard deviation.





13C



The bars on the box plots represent standard deviation. The black dots represent the outliers in the data set. The P-value was obtained by pairwise comparison of the dimension among isolates. The pairwise comparisons with significant P- values are significantly different from each other.

Ceratocystis fimbriata Syngonium isolates were morphologically identical on 10% V8 plates. The cultures were dark olive in color. Isolates 3401, 3421, 3459 and 3466 produced cylindrical endoconidia, doliform endoconidia and aleurioconidia (chlamydospore). They also produced ascospores on flask shaped perithecia. Cylindrical endoconidia were hyaline, produced on phialides and released in chains. The spores readily disintegrated from the chain and appeared separate under the microscope. Cylindrical endoconidia were the most abundant asexual spores. Doliform endoconidia were hyaline and released in the form of chains of spores. The chains did not disintegrate under the microscope. Doliform conidia were not as abundant as cylindrical endoconidia. Phialides producing doliform conidia were mostly embedded on the base of perithecia. Aleurioconidia were dark and pigmented, produced singly or in short chains of 2-6 spores. Perithecia were dark colored with a round to ovoid base, dark elongated neck and hyaline hyphae. Ascospores were hyaline and hat shaped and were released embedded in mucilage that formed a

cream-colored droplet on top of the perithecia. Perithecia were distributed randomly across the plates and began to form at 5 days on 10% V8 agar. The average and range of different spore dimensions for the four isolates are illustrated in Table 11.

Table 11. Average and range of different spore dimensions for *Ceratocystis fimbriata* isolates 3401, 3421, 3459 and 3466.

	3401	**3421**	**3459**	**3466**
Cylindrical endoconidia (mean)	28.69 x 4.90	30.90 x 4.71	30.86 x 4.75	33.12 x 4.70
Cylindrical endoconidia (range)	12.5 x 4 – 75 x 5.25	12.5 x 4 – 72 x 5.25	12.5 x 4 – 80 x 5.25	12.5 x 4 – 80 x 5.25
Doliform endoconidia (mean)	8.19 x 7.33	8.21 x 7.41	9.41 x 7.28	9.56 x 7.13
Doliform endoconidia (range)	6.68 x 5.84 – 10.85 x 10.02	7.51 x 6.68 – 10.02 x 8.35	8.35 x 6.68 – 10.85 x 10.02	8.35 x 6.68 – 10.85 x 8.35
Ascospore (mean)	6.9 x 5.25	6.57 x 4.88	6.59 x 4.88	6.70 x5.01
Ascospore (range)	5.01 x 3.34 – 8.35 x 6.68	5.01 x 3.34 – 7.51 x 6.68	5.01 x 3.34 – 8.35 x 6.68	5.01 x 3.34 – 8.35 x 6.68
Aleurioconidia (mean)	13.36 x 10.47	13.25 x 9.94	13.29 x 10.17	13.15 x 10.05
Aleurioconidia (range)	10.02 x 8.35 – 15.03 x 11.69	8.75 x 7.50 – 16.25 x 12.52	10.02 x 8.35 – 16.70 x 11.69	10.02 x 8.35 – 16.70 x 11.69

All measurements are in µm. Mean is expressed as length x width. Range is expressed as minimum length x minimum width – maximum length x maximum width.

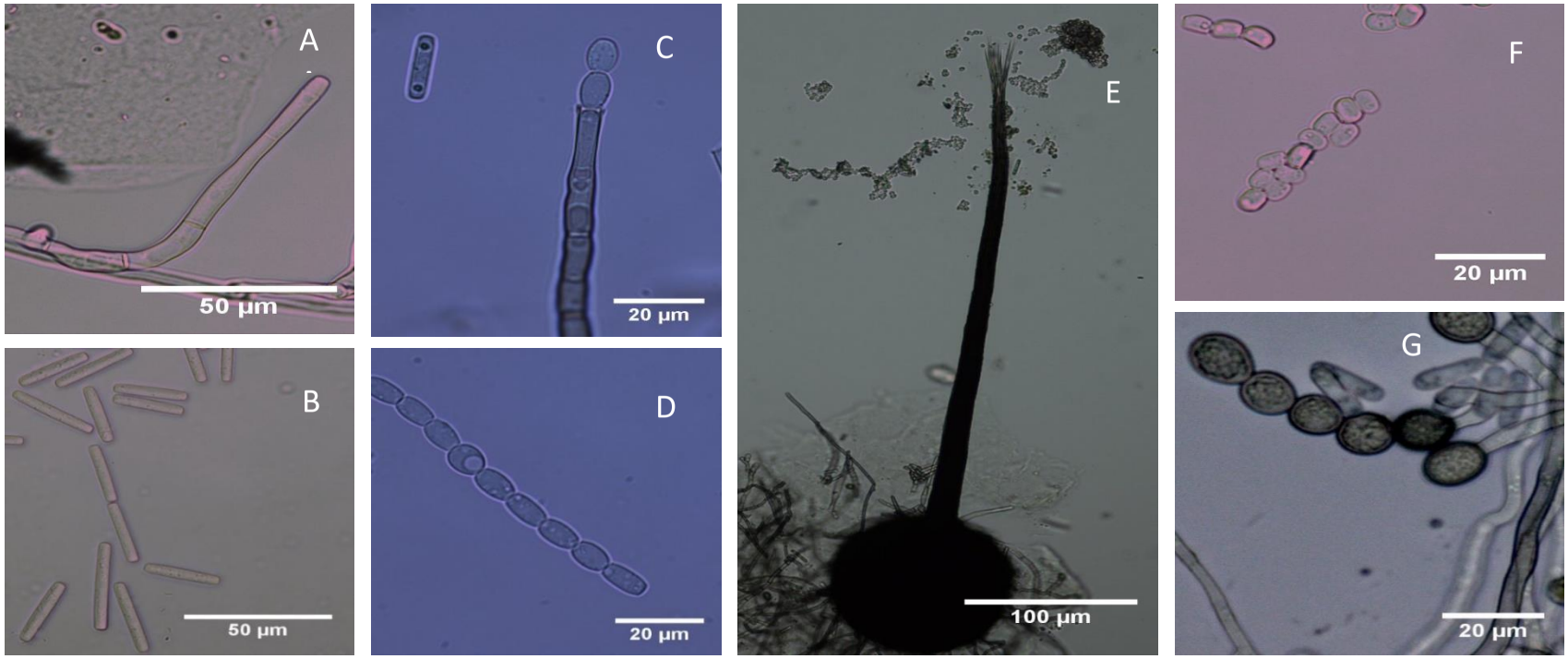


Figure 14. Conidiophores and spores of *Ceratocystis fimbriata*. A: phialide producing cylindrical endoconidia (400X magnification), B: Cylindrical endoconidia (400X magnification), C: phialide producing doliform endoconidia with two doliform endoconidia being released (600X magnification), D: Chain of doliform endoconidia (600X magnification), E: Perithecia (100X magnification), F: Ascospores (600X magnification), G: Aleurioconidia (chlamydospores) borne singly and in chain (600X magnification).

Discussion and Conclusion

Dimensions of ascospores, cylindrical endoconidia, doliform endoconidia, aleurioconidia and perithecia were not normally distributed. Thus, analysis of variance (ANOVA) for normally distributed data was not possible. Significance difference in the distribution of values among the groups was done using Kruskal Wallis test. When Kruskal Wallis test was significant Dunn test was performed for pairwise comparison. R package FSA (Ogle, D.H. 2018) was used for Dunn test and P-value were adjusted according to Benjamini-Hochberg method.

The three isolates of *C. fimbriata* (3401, 3459 and 3466) from *Syngonium* are similar on growth at 15°C, 20°C, 25°C and 30°C. The data was subjected to analysis of variance (ANOVA) at 0.05 level of significance, where they were found not to be statistically different. However, *C. fimbriata* isolates from *Syngonium* tend to grow faster compared to *C. lukuohia* and *C. huliohia* from ohia. The average growth rate for *C. lukuohia* and *C. huliohia* is reported to be 3.6 mm/day and 4.3 mm/day, respectively after 14 days (Barnes et al. 2018). The growth rates for *C. fimbriata* isolates 3401, 3466 and 3459 in this study were observed to be 5.17 mm/day, 5.17 mm/day and 5.21 mm/day. respectively. Optimum growth for *C. lukuohia*, *C. huliohia* and *C. fimbriata* from *Syngonium* occurred at 25°C. Webster and Butler (1967) reported that 25°C is optimal for growth of *C. fimbriata* isolates from sweet potato (*Ipomoea batatus*), cacao, coffee, plane tree, aspen, oak, apricot, prune, peach and almond.

Pairwise comparison of spore and perithecial dimensions revealed significant differences among some of the isolates (3401, 3421, 3666 and 3459) for cylindrical endoconidia length, doliform endoconidia length, ascospore length and width and perithecial length, width and neck length. However, analysis of the growth rate of isolates 3401, 3459, 3466 showed no statistical difference at 0.05 level of significance. Similarly, these four isolates had identical nucleotide sequences for five gene loci (discussed in detail in chapter five). Cylindrical endoconidia and ascospores of *C. fimbriata* isolates from *Syngonium* are longer and wider compared to the same dimensions reported for *C. lukuohia* and *C. huliohia.* The average

cylindrical endoconidial length and width for *C. lukuohia* isolates are 16 x 4 µm and 20 x 4 µm (Barnes et al. 2018), while we observed the average cylindrical length and width for *C. fimbriata* from *Syngonium* to be 28 - 33 µm x 4.7 - 4.9 µm in this study. Some of the *C. fimbriata* cylindrical endoconidia from *Syngonium* were much longer compared to others. The range for the cylindrical endoconidial length was 15-80 µm for isolate 3466. The average ascospore dimensions for *C. fimbriata* from *Syngonium* are 6.7-6.9 x 4.8-5.2 µm. Barnes et al. (2018) reported the ascospore dimensions for three *C. lukuohia* isolates as 5 x 3 µm, 5 x 3 µm and 5.5 x 3 µm, and the dimensions for *C. huliohia* as 5 x 4 µm. The difference in the measurements and growth rate could have arised due to the different media used. Ten percent V8 agar plates were used in this study while Barnes et al. (2018) used 2% MEA plates. *Ceratocystis uchidae* that infects *Colocasia esculenta* in Hawaii and *Xanthosoma* species in Fiji has smaller ascospores (4-6 x 2-4.5 µm), small perithecial bases (83-190 µm) and neck length (180-520 µm) compared to the perithecial base (180-229 x 182-237 µm) (Li et al. 2017) and neck length (629-834 µm) of *C. fimbriata* isolates from *Syngonium*. Perithecial base, perithecial neck length and ascospore dimensions for *C. fimbriata* isolates from *Syngonium* fall within the range described by Webster and Butler (1967) for *C. fimbriata* isolates. Aleurioconidia length and width for *C. fimbriata* isolates (13.1-13.3 x 9.9-10.4 µm) from *Syngonium* and *C. lukuohia* (13-14 x 10 µm) and *C. huliohia* (13x11 µm) from ohia appear to be the same. Analysis of the growth rate at different temperatures and identical sequences support the hypothesis that the four *C. fimbriata* isolates from *Syngonium* are identical. However, the analysis of different spore size says otherwise. Most likely all four isolates of *C. fimbriata* from *Syngonium* isolates are identical as supported by the gene sequences and analysis of growth rate at different temperatures. It is also possible that the differences among the isolates were not detected because the sequences were generated for only 5 gene loci, which are a very small portion of the genome.

CHAPTER 5

MOLECULAR CHARACTERIZATION OF *CERATOCYSTIS FIMBRIATA* ISOLATES FROM *SYNGONIUM*

Introduction

Phylogenetic analysis based on multiple gene loci in the genome is useful for classification and identification of new species based on the percentage nucleotide identity shared with other closely related pathogen species (Bridge et. al 2005), as well as to show the evolution. Phylogenetic analysis using one or more genes is also commonly used to study the genetic diversity within and among different plant pathogen species (He et al. 2017). *Ceratocystis lukuohia* is closely related to *C. fimbriata* from *Syngonium* (Barnes et. al 2018). There is no information available on the diversity of *C. fimbriata* isolates infecting *Syngonium* in Hawaii. Internal Transcribed Spacer (ITS) rDNA region, Beta-tubulin 1(*Bt1*), and Transcription elongation factor-1 alpha (*tef1*) are commonly used genes for phylogenetic studies of fungi, including *Ceratocystis* spp. Here, in an attempt to characterize the *C. fimbriata* isolates from *Syngonium*, we used two other gene loci (Guanine Nucleotide binding protein subunit beta-like protein (*ms204*) and second largest subunits of RNA polymerase II (*rpb2*) in addition to the above-mentioned gene loci used by Barnes et. al 2018.

Materials and Methods

DNA extraction

For DNA extraction, *C. fimbriata* cultures were grown in liquid plich media (Kamoun et al. 1993). Plich media was prepared by dissolving the contents listed in table 12 in distilled water to make the final volume 500 ml. All chemicals were added to water and stirred until fully dissolved. The medium was autoclaved for 30 minutes at 121°C and poured into 100mm x15mm petri plates. A small plug of mycelium was taken from actively growing cultures and transferred to the plates containing plich medium

and incubated under dark conditions to promote mycelial growth. Mycelium growing in liquid media was collected after 14 days by vacuum filtration. A thin sheet of mycelium collected on the surface of the filter paper was scraped off and crushed with liquid nitrogen to obtain a fine powder. About 100 mg of powder per isolate was used for DNA extraction using the DNeasy Plant Minikit (Qiagen, MD, USA) following manufacturer's instructions.

Table 12. Recipe for Plich medium.

Chemicals	Quantity
KH_2P0_4	0.25g
$MgS0_4$	0.125g
Glucose	12.5g
L-Asparagine	0.5g
Yeast extract	0.25g

Table 13. List of isolates used for molecular analysis and the location they were isolated from.

Identification Number	Collection location
3401	Novelty Greens, Hilo
3404	Novelty Greens, Hilo
3414	Novelty Greens, Hilo
3416	Novelty Greens, Hilo
3424	Novelty Greens, Hilo
3428	Novelty Greens, Hilo
3433	Novelty Greens, Hilo
3436	Novelty Greens, Hilo
3440	H. Eunice Nursery, Hilo
3443	H. Eunice Nursery, Hilo
3452	H. Eunice Nursery, Hilo
3458	H. Eunice Nursery, Hilo
3466	H. Eunice Nursery, Hilo
3470	H. Eunice Nursery, Hilo
3421	H. Eunice Nursery, Hilo

PCR and Sequencing

For phylogenetic analysis, five genes, Internal Transcribed Spacer (ITS) rDNA region, Beta-tubulin 1(*Bt1*), Transcription elongation factor-1 alpha (*tef1*), Guanine Nucleotide binding protein subunit beta-like protein (*ms204*) and second largest subunits of RNA polymerase II (*rpb2*) were selected. ITS rRNA region was amplified using ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3 (White et al. 1990). EFCF1 (5'-AGTGCGGTGGTATCGACAAG -3') and EFCF6 (5'-CATGTCACGGACGGCGAAAC -3') primers were used to amplify alpha subunit of elongation factor (Oliveria et al. 2015). PCR for beta tubulin gene was performed using primer βt1a (5` TTCCCCCGTCTCCACTTCTTCATG 3`) and βt1b (5' GACGAGATCGTTCATGTTGAACTC 3') (Glass and Donaldson 1995). Similarly, MS204F.cerato (AAG GGC ACC CTC GAG GGC CAC) and MS204R.cerato (GAT GGT RAC GGT GTT GAT GTA) primers were used for the amplification of guanine nucleotide-binding protein subunit beta-like protein (ms204 gene) (Fourie et al. 2014). RPB2-5Fb (GAY GAY CGT GAT CAC TTY GG) and RPB2-7Rb (CCC ATR GCY TGY TTR CCC AT) was used for the larger subunit of the RNA polymerase II (Fourie et al. 2014).

Gradient PCR was conducted for each gene to find the correct annealing temperature. Serial dilutions of DNA were done from 5ng/µl to 30ng/µl at 5ng/µl increment to find the optimal DNA concentration for PCR. PCR was conducted in 50 µl total volume. Each PCR reaction consisted of 2 µl each of forward and reverse primer (10 µm concentration), 25 µl of DNAse and RNAse free water (Invitrogen, Carlsbad, CA), 20 µl of ImmoMix (Bioline, Mephis, TN) or Mytaqred (Bioline, Mephis, TN) mastermix and 1 µl genomic DNA (10 ng/µl). 1 µl of sterile DNAse and RNAse free water was used instead of Genomic DNA in negative control tubes. PCR reaction was conducted in Mastercycler^{R} (Eppendorf, Germany). PCR conditions for each of the five genes are listed in the table below.

14. PCR conditions for the primers used.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Number of Cycles
ITS	95°C for 5 min	95°C for 30 sec	50°C for 1 min	72°C for 1 min	72°C for 3 min	30
Bt1	95°C for 10 min	95°C for 30 sec	53°C for 1 min	72°C for 1 min	72°C for 3 min	35
tef1	95°C for 3 min	95°C for 30 sec	60°C for 30 sec	72°C for 1 min	72°C for 2 min	30
ms204	95°C for 10 min	95°C for 30 sec	58°C for 50 sec	72°C for 1 min	72°C for 5 min	30
rpb2	95°C for 10 min	95°C for 30 sec	52°C for 30 sec	72°C for 1 min	72°C for 3 min	35

PCR products obtained were electrophoresed in 1.5% agarose gels for 80 minutes at 100 volts and visualized under UV light to check for the amplification of the targeted genes. Once the amplification of the genes was confirmed the PCR product was purified. For purification, 5µl of PCR product was mixed with 2 µl of ExoSap-IT (Thermofisher Scientific, Waltham, MA) and incubated at 37°C for 15 minutes followed by incubation at 80°C for 15 minutes. One µl each of forward and reverse primers were added to 2.5 µl of the cleaned PCR product in two tubes and diluted with sterile water to make final volumes of 15 µl. PCR products were sequenced in both direction at Genewize Inc (La Jolla, CA).

Phylogenetic Analysis

The forward and reverse sequences obtained after sequencing were aligned using Geneious version 10.2.3 and manually edited to correct the sequencing errors when present followed by multiple alignment of consensus sequences of each of five genes. Genes *Bt1, tef1*, *rpb2* and *ms204* were used for phylogenetic analysis and sequences of *Ceratocystis* species closely related to *C. lukuohia* and *C. huluohia* were obtained from NCBI GeneBank. Accession numbers were obtained from Barnes et al. (2018). *Ceratocystis fimbriata* isolates 3401, 3421 and 3466 from this study were selected to include in the phylogenetic tree. Neighbor joining tree was constructed using MEGA 7 (Kumar et al. 2016) and edited using PowerPoint. Support for the branches were calculated by 1000 bootstrap replicates. *Ceratocystis albifundus* was used as outgroup. ITS region could not be used in the phylogenetic analysis because the ITS sequences for *Metrosideros polymorpha* and *Ipomea batatus* isolates were not available in the GeneBank.

Results

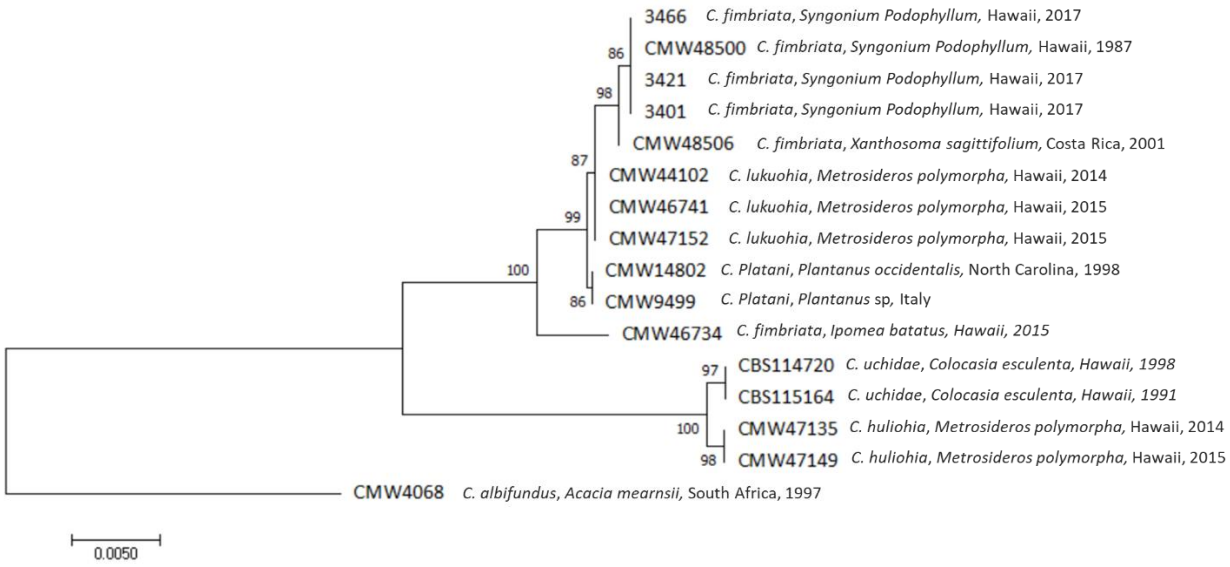
Ceratocystis fimbriata isolates grown for 14 days produced ~1-gram mycelium. The DNA obtained from DNeasy Plant Minikit was of high quality and the concentration ranged from 30 to 225 ng/µl. Upon diluting the DNA to determine the right concentration for PCR, no difference in the intensity of the bands was observed for 5, 10, 15, 20, 30, 40 or 50 ng/µl of DNA per reaction. Thus, DNA for all isolates was diluted to 10 ng/µl and 1 µl of it was used per reaction. Non-specific bands were observed for Transcription elongation factor-1 alpha (*tef1*) with the primers used. To avoid non-specific amplification different temperature and time combinations were tested for annealing, final extension and number of cycles. A single cycle of initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 2 min gave a single specific band for this gene. Poor quality sequences were obtained with the reverse

primer (RPB2-7Rb) for the gene guanine- nucleotide binding protein (*rpb-2*) on multiple attempts. Thus, only the forward sequences were analyzed for *rpb-2*. The quality of the sequences was very good otherwise.

Trimming of the poor-quality ends resulted in 565 bp, 553 bp, 1358 bp, 1350 bp and 926 bp long sequences for the genes *ITS, Bt1, tef1, ms204,* and *rpb2,* respectively which were compared against the sequences available in the NCBI gene bank to establish the identity of the sequences. All 15 isolates had identical sequences for all five genes.

For phylogenetic analysis, concatenation of the *Bt1, tef1, ms204,* and *rpb2* sequences gave 3014 bp long fragment. *Ceratocystis fimbriata* isolates collected in this study (3401, 3421 and 3466) had identical sequences to isolate (CMW48500 or CBS114719) which was collected in 1987 and they grouped together in phylogenetic analysis. *Ceratocystis fimbriata* from *Xanthosoma sagittifolium* was different from *C. fimbriata* from *Syngonium* at two nucleotide positions (one in *rpb2* and other in *tef1* gene) and formed a sister clade to *C. fimbriata* isolates from *Syngonium*. *Ceratocystis fimbriata* from *Syngonium* were different from *C. lukuohia* isolates at 6 nucleotide position and were identical for *Bt1* gene. *Ceratocystis lukuohia* had identical sequences to *C. fimbriata* from *Xanthosoma sagittifolium* except at four nucleotide position and had identical sequences for *Bt1* and *tef1* genes. *Ceratocystis fimbriata* from *Syngonium* and *C. platani* were different at nine nucleotide position. *Ceratocystis lukuohia* was most close to *C. platani* in phylogenetic analysis and they differed at 3 nucleotides positions.

Figure 15. Neighbor joining tree based on the combined sequences for *Bt1*, *tef1*, *ms204* and *rpb2*.



Discussion and Conclusion

The two populations of *C. fimbriata* obtained from *Syngonium* in Hilo, Hawaii Island are clonal. There was no nucleotide difference among the sequences from the 15 isolates for the genes Internal Transcribed Spacer (ITS) rDNA region (ITS), Beta-tubulin 1(*Bt1*), Transcription elongation factor-1 alpha (*tef1*) Guanine Nucleotide binding protein subunit beta-like protein (*ms204*) and Second largest subunits of RNA polymerase II (*rpb2*). The two populations were isolated from samples collected from two commercial nurseries in Hilo, Hawaii, owned by relatives. Sequences for Internal Transcribed Spacer rDNA region (ITS) for the isolates collected for the study were identical to the sequences of the isolates collected in Hawaii in 1979 and 2017. This suggests the pathogen has undergone no genetic changes or the same clonal population is being repeatedly introduced in infected planting materials. *Syngonium* isolates formed a sister clade with *C. lukuohia* infecting ohia and they are in the Latin American clade of *C. fimbriata* (Barnes et al. 2018). Nursery owners import the rooted cuttings from Florida. ITS sequences of *C. fimbriata* from Florida, Brazil, and Australia and the isolate CBS 114719 (strain collected from

Hawaii in the 1980Ss are identical (Thorpe et al. 2005). Plant pathogens easily move from one geographic location to another (Baker et al. 2003; Harrington et al. 2015) and this appears to be the case for *C. fimbriata* from aroids (Thorpe et al. 2005). *Colocasia* isolates from China had ITS sequences identical to *Colocasia* isolates from Hawaii and *Xanthosoma* isolates from Fiji, as well as *Xanthosoma* isolates from Puerto Rico, Cuba, Dominician Republic and Costa Rica (Thorpe et al. 2005).

Ceratocystis lukuohia isolates were most closely related to *C. platani* isolates in the neighbor joining tree we constructed but Barnes et al. (2018) report *Ceratocystis lukuohia* isolates to be most closely related to *C. fimbriata* isolates from *Xanthosoma sagittifolium* and *Syngonium* sp. This could be due to the shorted rpb2 sequences used in this study which had higher power to resolve these species.

OVERALL CONCLUSION

Ceratocystis fimbriata from *Syngonium* has restricted distribution in Hawaii. *Ceratocystis fimbriata* isolates 3401, 3421, 3459 and 3466 were statistically different to each other in terms of the dimensions of perithecia, sexual and asexual spores. However, isolates 3401, 3459 and 3466 did not differ in radial growth at 15°C, 20°C, 25°C and 30°C. In addition, the fifteen isolates collected from *Syngonium* in this study were genetically identical for the five genes sequenced. Thus, most likely a single strain of *C. fimbriata* is present at both nurseries in Hilo. *Ceratocystis fimbriata* from *Syngonium* is not pathogenic to ohia.

Ceratocystis fimbriata isolates originally collected by Dr. Janice Uchida in the 1980s, and isolates collected in this study had identical sequences for ITS, *Bt1* and *tef1*. Thus, it appears that *C. fimbriata* from *Syngonium* in Hawaii has undergone no genetic modification since it was first introduced.

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